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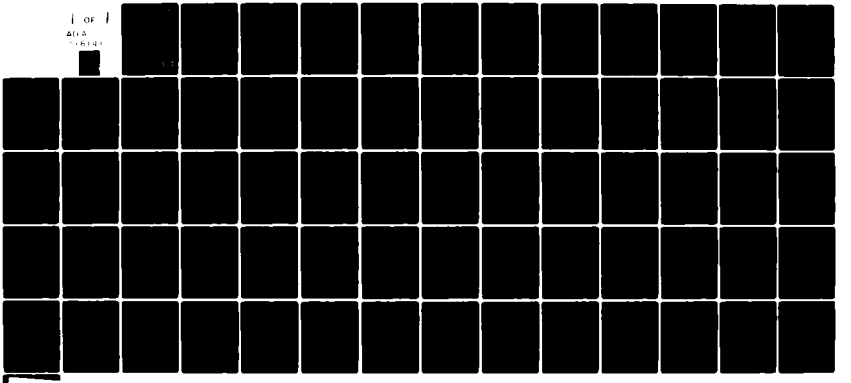

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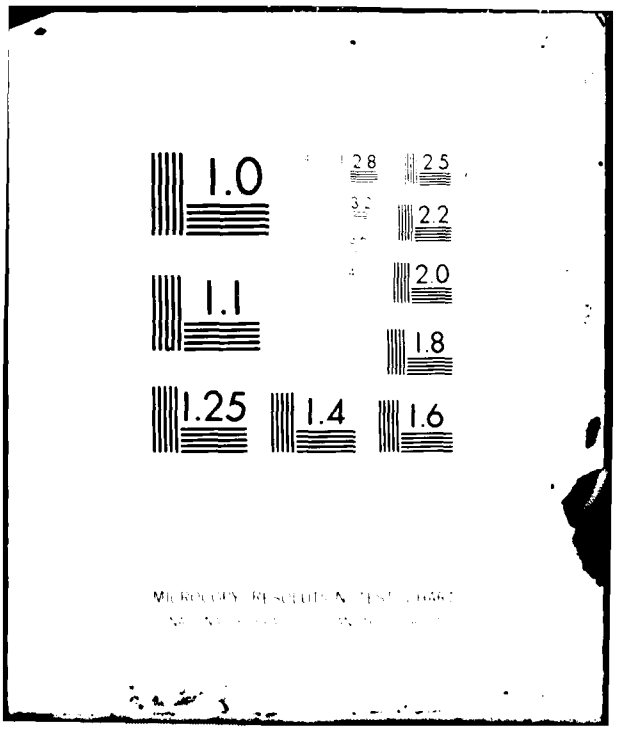
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REPORT #5

Host Defense Against Opportunist Microorganisms Following Trauma

ANNUAL SUMMARY REPORT

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SEPTEMBER, 1980

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick
Frederick, Maryland 21701

Contract No. DAMD-17-76-C-6023

University of Cincinnati

Cincinnati, Ohio 45221

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS *BEFORE COMPLETING FORM*
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A116 242	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* HOST DEFENSE AGAINST OPPORTUNIST MICROORGANISMS FOLLOWING TRAUMA		5. TYPE OF REPORT & PERIOD COVERED Annual Summary Report No. 5, 8/1/79 to 9/30/80
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Ann B. Bjornson, Ph.D. H. Stephen Bjornson, M.D., Ph.D. William A. Altemeier, M.D. Josef E. Fischer, M.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD 17-76-C-6023
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of Cincinnati Cincinnati, Ohio 45267 College of Medicine		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A. 3A161102BS05.00.039
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE September, 1980
		13. NUMBER OF PAGES 69
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

burn	polymorphonuclear leukocyte	septicemia
trauma	immunoglobulins	microorganisms
injury	opsonin	infection
complement	phagocytosis	candidosis
antibodies	opportunist	candidemia

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Studies were performed to further investigate two abnormalities of host defense in burned patients, i.e., serum-mediated inhibition of polymorphonuclear leukocyte bactericidal activity and reduction in alternative complement pathway mediated C3 conversion. In addition, studies were initiated to determine the association between colonization of body surfaces with Candida, Candida antigenemia, and systemic candidosis. Serum-mediated inhibition of polymorphonuclear leukocyte bactericidal activity was demonstrated in 3 of 12 burned

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Unclassified

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

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patients during 13 to 56 days postburn. Decreased bactericidal activity was related to an inhibitory effect of the burn sera on the phagocytic process, which was not associated with cell death. The reduction in alternative pathway mediated C3 conversion was demonstrated in 18 burned patients, with the most marked reduction occurring in patients with large full-thickness injuries and infectious complications. Evidence was provided to suggest that this abnormality was caused by a dialyzable low molecular weight inhibitor. Systemic candidosis occurring in 2 of 8 hyperalimented burned patients was associated with catheter and catheter insertion site colonization with Candida and Candida antigenemia as detected by the inhibition enzyme-linked immunosorbent assay. The mouth appeared to be the primary reservoir for Candida providing a source of Candida for colonization of the catheter insertion site.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, 1978].

SUMMARY

Studies were performed to further investigate two abnormalities of host defense in burned patients, i.e., serum-mediated inhibition of polymorphonuclear leukocyte bactericidal activity and reduction in alternative complement pathway mediated C3 conversion. In addition, studies were initiated to determine the association between colonization of body surfaces with Candida, the presence of Candida antigenemia, and the occurrence of systemic candidosis.

Serum-mediated inhibition of polymorphonuclear leukocyte bactericidal activity was demonstrated in 3 of 12 burned patients during 13 to 56 days postburn. Decreased bactericidal activity was related to an inhibitory effect of the burn sera on the phagocytic process. The inhibitory effect was shown to involve a direct interaction of the burn sera with the leukocytes, which was not associated with cell death and was not reversed by washing of the leukocytes. The reduction in alternative pathway mediated C3 conversion was demonstrated in 18 patients with total burn sizes of 20% or greater, with the most marked reduction occurring in patients with large full-thickness injuries and infectious complications. Dialysis of the burn sera was shown to correct the reduction in C3 conversion, suggesting that this abnormality was caused by a dialyzable low molecular weight inhibitor. In contrast, the burn serum inhibitor of polymorphonuclear leukocyte phagocytosis was shown to be non-dialyzable. It is postulated that the burn serum inhibitors, although distinct from each other, may mediate multiple host defense abnormalities.

Systemic candidosis occurring in 2 of 8 hyperalimented burned patients was associated with catheter and catheter insertion site colonization with Candida and Candida antigenemia as detected by the inhibition enzyme-linked immunosorbent assay. The mouth appeared to be the primary reservoir for Candida providing a source of Candida for colonization of the catheter insertion site. Rectal and burn wound colonization were not associated with the development of systemic candidosis unless concurrent catheter and catheter insertion site colonization were documented.

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B. To provide additional experimental evidence to confirm the observation that the inhibition of PMN bactericidal activity is not caused by deficient opsonization of the bacteria by the burn sera.

C. To determine if the inhibition of PMN bactericidal activity is related to an effect of the burn sera on phagocytosis or intracellular killing or both processes.

D. To determine if the inhibition of PMN bactericidal activity is related to a toxic effect of the burn sera on the leukocytes.

E. To reinvestigate the relationship between the reduction in alternative complement pathway mediated C3 conversion, burn size, and the occurrence of bacteremia in burned patients.

F. To partially characterize the inhibitor of C3 conversion in burn sera.

G. To determine the temporal relationship between colonization of body surfaces with Candida and the development of systemic candidosis in burned patients receiving total parenteral nutrition (TPN).

H. To evaluate the diagnostic efficacy of the enzyme-linked immunosorbent assay (ELISA) for the early detection of systemic candidosis in burned patients receiving TPN.

II. BACKGROUND

A. Inhibitory effect of burn sera on PMN bactericidal activity

In our previous studies to determine the opsonic activity of bacteremic burned patients' sera for their infecting bacterial strains (1-3), an in vitro bactericidal assay consisting of combinations of bacteria, serum, and normal human PMNs was utilized. The serum concentrations ranged from 1% to 10% and were based on the minimal concentration of pooled normal human serum which promoted optimal phagocytosis and intracellular killing of each bacterial strain by the leukocytes. This experimental approach maximized the possibility of demonstrating decreased opsonic activity in the burn sera. However, reduction in opsonic activity was demonstrated in only 3 of 28 patients.

Before ruling out the possibility that burn sera had an inhibitory effect on PMN bactericidal activity, phagocytosis and intracellular killing of bacteria by normal human PMNs was measured in the presence of a physiologic concentration of burn serum or pooled normal human serum. The results were compared to those obtained from assays in which 10% of the sera was employed.

These preliminary experiments were performed on a 3-year-old female with 40% total body surface burn and 33% third degree injury, who had bacteremia caused by Staphylococcus aureus during her clinical course. Because of the need to use large quantities of sera in the assays, serial serum samples obtained from the patient at weekly intervals were pooled. Prior to testing, the pooled burn serum and normal serum were dialyzed for 18 hours at 4°C against 0.01 M phosphate buffered saline, pH 7.4, containing 1.5×10^{-4} M $CaCl_2$ and 5.0×10^{-4} M $MgCl_2$ (PBS^{2+}) using 12,000 molecular weight retention dialysis tubing. The dialysis procedure was performed to insure that antibiotics, which would result in killing of the bacteria in the absence of PMNs, were removed from the burn serum.

The patient's serum promoted normal phagocytosis and killing of her infecting S. aureus strain by the PMNs when tested at a concentration of 10%. However, when the concentration of sera in the bactericidal assays was increased to 98%, the patient's serum was unable to support phagocytosis and killing of the bacteria by the PMNs. The normal serum promoted over a 1-log reduction in bacterial counts by the PMNs at concentrations of 10% and 98%. Killing of the bacteria was not demonstrated in the presence of patient's or normal serum in the absence of PMNs, or by PMNs in the absence of serum. These results indicated that the patient's serum had an inhibitory effect on PMN bactericidal activity when tested at a physiologic concentration.

To determine if the inhibition of PMN bactericidal activity was related to deficient opsonization of the bacteria by the burn serum, the bacteria were incubated with 98% of burn serum or normal serum, washed, and then tested for their ability to be phagocytosed and killed intracellularly by the PMNs. Bacteria opsonized with the patient's serum were phagocytosed and killed as efficiently as bacteria opsonized with the normal serum. Phagocytosis and killing of bacteria opsonized with diluent without serum was not demonstrated. These results suggested that the inhibition of PMN bactericidal activity was not related to deficient opsonization of the bacteria by the burn serum.

B. Reduction in alternative complement pathway mediated C3 conversion following burn injury

In 1976, we reported that C3 conversion by inulin, a recognized activating substance of the alternative complement pathway, was significantly reduced in multiple serum samples obtained from 5 burned patients in comparison to C3 conversion by inulin in normal human sera (4). Our subsequent studies on a total of 50 burned patients confirmed the existence of this abnormality in patients with burns involving greater than 45% total body surface (1-3,5). Reduction in C3 conversion in these patients was demonstrated during the early acute burn phase and was generally normalized by the seventh postburn week (1-3,5). The duration of the reduction in C3 conversion was correlated with increasing burn size (5). Decreased C3 conversion occurred with the same frequency in young and older burned patients and in bacteremic and non-bacteremic patients (1-3,5).

Extensive studies have been performed in our laboratory to determine the mechanism of the reduction in alternative complement pathway mediated C3

conversion in burn sera. Our initial experimental approach to this problem was to determine the ability of pooled normal human serum to restore C3 conversion in the burn sera. Mixture of equal parts of burn sera with reduced C3 conversion and pooled normal human serum did not restore C3 conversion to normal (4,5). These preliminary results suggested that reduction in C3 conversion in the burn sera might be caused by a circulating inhibitor.

The hypothesis that reduction in C3 conversion in the burn sera was caused by an inhibitor was tested experimentally (Annual Summary Report, June, 1977). Burn sera with normal or reduced C3 conversion were tested for their ability to inhibit C3 conversion in pooled normal human serum. None of the burn sera with normal C3 conversion inhibited C3 conversion to any extent when added in increasing concentrations to the normal serum. Several of the burn sera with reduced C3 conversion inhibited C3 conversion when added to the normal serum, whereas other burn sera with reduced C3 conversion had no inhibitory activity. When the grouped data were subjected to statistical analysis, the differences in inhibition of C3 conversion by burn sera with reduced versus normal C3 conversion were not found to be significant. In addition, the values obtained when equal parts of burn sera and pooled normal human serum were added together were not considerably lower than those obtained when complement deficient control sera were added to pooled normal human serum. These results suggested that reduction in C3 conversion in the burn sera might be caused by a deficiency of serum proteins, by an inhibitor of C3 conversion which had been diluted by mixture with normal serum, or by elevation of normal regulatory proteins of the alternative complement pathway, β1H or C3b inactivator (C3bINA).

Subsequent studies failed to demonstrate a correlation between reduction in C3 conversion and elevation of β1H or C3bINA, or both proteins, in multiple burn sera (Annual Summary Report, June, 1978). This observation suggested that reduction in C3 conversion was not caused by elevation of these regulatory proteins.

In all of the studies described above, alternative pathway mediated C3 conversion had been measured in sera which had been tested concurrently for opsonic activity. Because antibiotics had to be removed from the sera prior to use in the opsonic assays, the sera had been dialyzed for 18 hours at 4°C against PBS^{2+}. During the last contract period, C3 conversion in non-dialyzed and dialyzed burn sera was compared. The results showed that dialysis using 3,500 or 12,000 molecular weight retention dialysis tubing partially corrected the reduction in C3 conversion (Annual Summary Report, June, 1979). A second dialysis of the burn sera further improved C3 conversion. Incubation of the burn sera at 4°C for 18 hours without dialysis did not affect C3 conversion. These results suggested that the reduction in C3 conversion might be caused by a dialyzable inhibitor with a molecular weight of less than 3,500 daltons.

Since magnesium ions are known to be required for alternative complement pathway mediated C3 conversion, the possibility was considered that an abnormal concentration of magnesium ions in the burn sera might have caused the reduction in C3 conversion. Dialysis of the sera would have

standardized the concentration of magnesium ions, perhaps providing the ionic environment required for normal C3 conversion. However, the concentration of magnesium ions in non-dialyzed burn sera was found to be normal, suggesting that reduction in C3 conversion was not caused by an abnormal concentration of these ions.

The observation that dialysis of burn sera improved C3 conversion suggested that reduction in C3 conversion in certain burned patients was probably missed in our previous studies, in which only dialyzed sera were tested. This is perhaps most relevant to patients with burns involving less than 45% total body surface, who were previously shown to have no demonstrable reduction in C3 conversion (5). In addition, the use of dialyzed sera in our previous investigations precludes any conclusions regarding the relationship between the duration of the reduction in C3 conversion, burn size, and the occurrence of bacteremia. Moreover, the inability to demonstrate a significant inhibition of C3 conversion by supplementation of normal serum with burn sera might also have been related to the use of dialyzed burn sera which lacked a full component of inhibitory activity.

C. Occurrence of Candida infections in burned patients and serodiagnosis

In recent years, wound and disseminated infections caused by Candida have emerged as a major problem in burned patients (6-10). Multiple factors have contributed to the increased incidence of infections caused by this opportunistic pathogen. The widespread use of topical and systemic antibiotics, while leading to better control of bacterial infections, may predispose the burned patient to fungal infections by altering the autochthonous microbial flora (6,11). Advances in the treatment of patients with severe burns have resulted in the survival of patients with large tissue defects, which may serve as portals of entry for Candida (9,10). The use of peripheral intravenous catheters and, more recently, central catheters for the administration of TPN has also been associated with an increased incidence of Candida infection in the burned (12,13) and unburned patient (14,15).

Little is known regarding the pathogenesis of Candida burn wound infections and disseminated disease caused by this fungus in the burned patient. Although Candida are distributed ubiquitously throughout the environment (16), the most important source of Candida in human infections appears to be man himself (17). Of the many sites in the body in which Candida has been demonstrated, the digestive tract appears to be the most frequent and clinically important habitat (18). From this source, the yeasts may readily colonize areas of the skin, the respiratory tract, or urogenital tract that are favorable to their multiplication. The ubiquity of Candida in the normal human microflora provides a natural reservoir of these fungi, which are able to cause infection when host defenses are impaired as is the case in the burned patient.

In systemic forms of candidosis, clinical evidence suggests that initial access to deep organs is usually gained by the hematogenous route

(18-20). Persorption of Candida albicans from the gut into the bloodstream has been demonstrated in monkeys, dogs, germ-free mice, and a human volunteer (20-22). This route appears to be important only when the numbers of Candida exceed a threshold, which has been shown to vary among animal species and may be lowered in debilitated subjects (20,21). Stone has demonstrated that intact skin and granulating wounds provide an effective barrier to invasion by C. albicans (19). Violation of the integrity of the skin or granulating wounds by intravenous catheters may provide Candida with a direct conduit into the circulation. If the infected catheter is removed early, the fungemia will be self-limiting in many cases (23,24). However, it is not known whether a threshold number of Candida colonizing a catheter insertion site are required before invasion of the bloodstream occurs. Data regarding the numbers of Candida at the catheter insertion site associated with bloodstream invasion would prove useful in monitoring burned patients, who often require multiple catheters over prolonged periods of time for fluid resuscitation as well as for nutritional support.

The rapid diagnosis of systemic candidosis is essential, since patients with compromised host defenses may die within a short period of time unless anti-Candida chemotherapy is instituted (25). The antifungal agent which has been shown to be most effective for therapy of systemic candidosis is amphotericin B, a drug that is so toxic that a firm diagnosis of candidosis should be established before treatment with this drug is initiated (26). However, clinical and mycological features of deep-seated forms of candidosis alone frequently provide evidence that is too equivocal for a definitive diagnosis (27).

The problems associated with establishing a firm diagnosis of systemic candidosis have stimulated the search for non-invasive techniques for accurate diagnosis. A variety of serologic tests have been developed for the detection of antibodies to Candida in the sera of patients with systemic candidosis (27,28). Counterimmunoelectrophoresis utilizing Candida "cytoplasmic" antigens for detecting specific antibodies is currently widely used for diagnostic purposes (27). However, Kozinn et al., in an analysis of their own and other data, showed that the sensitivity of this assay ranged from 27% to 100%; its specificity ranged from 57% to 96%, and its predictive value ranged from 20% to 90% (29). The low figures in this analysis were attributed to the lack of standardization in both the test methodology and the antigens used. The usefulness of serologic tests for the detection of anti-Candida antibodies in the diagnosis of candidosis is further compromised by false positive reactions (27).

An alternative approach to the diagnosis of systemic candidosis involves the chemical or immunologic detection of fungal cell antigens or metabolites in patients' sera. Crossed immunoelectrophoresis (30), a hemagglutination inhibition assay (31), gas-liquid chromatography (32,33), radioimmunoassay (34,35), and ELISA (36-39) have been used for the detection of Candida antigens in establishing a diagnosis of systemic candidosis in patients with various diseases; however, these tests have not yet been evaluated in the burned patient. The use of an enzyme as a marker gives the ELISA a sensitivity comparable to that of radioimmunoassay, but with stable reagents and simple equipment required for analysis (40). In addition, the ELISA is suitable for automation, which would be an advantage

in routine diagnostic work. Moreover, detection of circulating antigen by the ELISA or other methods does not depend on the host's immunologic responsiveness, which may be severely depressed in the burned patient.

III. EXPERIMENTAL APPROACH

Studies in the three areas of burn research which were presented in the preceding section of this report were to be performed during the project period. Fifteen to 20 burned patients were to be studied. Sera were to be collected weekly on all of the patients for 6 to 8 weeks post-burn. During episodes of bacteremia, sera were to be obtained one additional time per week. Bacteremic patients were to be included in the studies on serum-mediated inhibition of PMN bactericidal activity, and both bacteremic and non-bacteremic patients were to be included in the studies on the reduction in alternative complement pathway mediated C3 conversion. The studies on colonization with Candida and serodiagnosis were to be performed on 10 burned patients receiving TPN.

To determine the occurrence and duration of the inhibitory effect of burn sera on PMN bactericidal activity, serial serum samples from each bacteremic burned patient were to be tested at a physiologic concentration for their ability to support phagocytosis and intracellular killing of that patient's infecting bacterial strain by normal human PMNs in comparison to pooled normal human serum. Patients whose sera inhibited PMN bactericidal activity were to be included in subsequent experiments to determine if the inhibition was related to an effect of the burn sera on the opsonization, phagocytosis, or intracellular killing processes or to a toxic effect of the burn sera on the leukocytes. Individual sera which were shown to inhibit PMN bactericidal activity were to be pooled on each patient. Each patient's infecting bacterial strain was to be incubated with a physiologic concentration of that patient's pooled serum or pooled normal human serum. The bacteria were to be washed and then tested for their ability to be phagocytosed and killed intracellularly by normal PMNs. Extracellular and total surviving bacterial counts were to be compared in reaction mixtures consisting of a physiologic concentration of each patient's pooled serum, normal PMNs, and that patient's infecting bacterial strain. In addition, normal PMNs were to be incubated with a physiologic concentration of each patient's pooled serum, and viability was to be measured by trypan blue dye exclusion.

To reinvestigate the relationship between the reduction in alternative complement pathway mediated C3 conversion, burn size, and the occurrence of bacteremia, C3 conversion by inulin and cobra venom factor (CoVF) was to be measured in non-dialyzed serial serum samples obtained from the bacteremic and non-bacteremic burned patients. Patients with burns involving less than 45% total body surface were to be included in this aspect of the investigation for the purpose of documenting the presence or absence of the abnormality in this patient population.

The inhibitor of C3 conversion in burn sera was to be partially characterized by the following series of experiments. Individual sera which were shown to have reduced C3 conversion were to be pooled on each of 5 patients. Each patient's pooled serum was to be dialyzed at 4°C for 18

hours against PBS^{2+} or incubated at 4°C for 18 hours without dialysis. C3 conversion by inulin and CoVF was to be measured in the burn sera and compared to C3 conversion in identically treated pooled normal human serum. The dialysates from the burn sera and the normal serum were to be lyophilized, redissolved in distilled water, and chromatographed on Sephadex G-25. Fractions of the dialysates containing protein were to be added to pooled normal human serum and the dialyzed burn serum from which each dialysate had been prepared, and C3 conversion by inulin and CoVF was to be measured. Fractions of the dialysates which inhibited C3 conversion in the normal serum and in the dialyzed burn sera were to be pooled, lyophilized, and partially characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The temporal relationship between colonization of body surfaces with Candida and the development of systemic candidosis in burned patients receiving TPN was also to be investigated. Quantitative cultures for Candida were to be obtained from each patient prior to the insertion of the central venous catheter and after TPN was terminated. The body fluids which were to be cultured were blood and urine. In addition, cultures were to be obtained from the following sites: burn wound, rectum, oropharynx, and anterior nares. Cultures were also to be obtained from the skin of each patient at the site of insertion of the central venous catheter. Upon removal of the central catheter from each patient, quantitative cultures of segments of the catheter were also to be performed. During episodes of suspected septicemia, all of the cultures described above were to be repeated. Episodes of candidemia or evidence of systemic candidosis were to be correlated with changes in the numbers of Candida isolated from the serial quantitative cultures described above.

Antisera directed against three Candida antigens were to be prepared in rabbits and used for the detection of these antigens by the ELISA in serial serum samples obtained from the burned patients receiving TPN. The three antigen preparations were to be as follows: (a) crude homogenate of washed cells, (b) purified peptidoglucomannan, and (c) partially purified cytoplasmic fraction, lacking mannan. The three antisera were to be compared for their sensitivity and specificity for the detection of Candida antigenemia prior to and during candidemia and systemic candidosis. The presence of Candida antigenemia was also to be correlated with relative changes in the numbers of Candida isolated from the various body surfaces and fluids.

IV. PROGRESS REPORT

A. Studies to further Investigate the Inhibitory Effect of Burn Sera on PMN Bactericidal Activity.

1. Results

The study population consisted of 12 bacteremic burned patients. The group included the 7 bacteremic burned patients without underlying diseases who were followed during the contract period in the Cincinnati General Hospital and 5 additional bacteremic burned patients from the Shriners Burn Institute. The patients ranged in age from 4 to 81 years; nine were males, and three were females. All patients had flame burn injuries with the total burn sizes ranging from 23% to 80% with 10% to 75% third degree injury. The specific clinical characteristics of each patient and the postburn days of study are presented in Table 1.

All of the patients had one or more episodes of bacteremia caused by S. aureus. In six of the patients, bacteremia caused by other microorganisms was also documented. Pneumonia was demonstrated in seven of the patients and was the cause of death in four of them. Only one of the other eight patients did not survive; the cause of death in this patient was pulmonary edema. The nutritional requirements of eight of the patients were supplemented by peripheral alimentation with 10% dextrose in water plus 4.5% Free Amine and daily infusions of Intralipid. The temporal sequence of bacteremia, pneumonia, and peripheral alimentation in each patient is shown in Table 2.

Clotted blood specimens were drawn upon admission and at weekly intervals thereafter. During episodes of bacteremia, blood was drawn one additional time per week until blood cultures became negative. The blood specimens were allowed to clot for 1 hour at room temperature and were centrifuged at 5,000 g for 10 minutes at 4° C. The sera were removed, divided into small aliquots, and frozen at -70° C. Normal human sera were collected from 25 healthy adult donors and stored as a pool at -70° C. Prior to testing in the PMN bactericidal assays, all sera were dialyzed at 4° C for 18 hours against PBS^{2+} using 12,000 molecular weight retention dialysis tubing. The dialysis procedure was performed to remove antibiotics from the burn sera.

The PMN bactericidal assays were performed by a modification of the method of Hirsch and Strauss (41). Normal human PMNs were prepared by dextran sedimentation of heparinized whole blood from healthy adult donors followed by hypotonic lysis to remove remaining erythrocytes (42). The leukocytes were washed twice and resuspended in Hank's balanced salt solution containing 0.1% gelatin (HBG) to a final concentration of 1.0×10^7 PMNs/ml. Prior to each experiment, a frozen culture of the specific infecting S. aureus or Escherichia coli 075 was inoculated into a tube containing 5 ml of brain heart infusion broth, and the tube was incubated at 37° C for 18 hours. The bacteria were washed twice and resuspended in HBG to a final concentration of 1.0×10^8 cells/ml. Four hundred µl of the leukocyte suspension containing 4.0×10^6 PMNs were

Table 1. Ages, Sex, and Burn Sizes of the Patients and Postburn Days of Study.

Patient No.	Age	Sex[a]	Body Surface Injured (%)[b]		Postburn Days of Study
			Total	Third Degree	
1	6	M	80	75	6 - 26
2	12	M	75	75	21 - 56
3	18	M	54	20	21 - 61
4	21	M	51	47	2 - 53
5	11	M	49	12	14 - 46
6	4	F	48	48	17 - 52
7	42	M	41	17	5 - 20
8	49	M	33	10	15 - 40
9	14	M	32	24	8 - 32
10	38	F	28	10	3 - 56
11	81	F	24	14	5 - 52
12	77	M	23	22	6 - 56

[a] M = male; F = female.

[b] All patients had flame burn injuries.

Table 2. Temporal Sequence of Bacteremia, Pneumonia, and Peripheral Alimentation in the Patients.

Patient No.	Bacteremia[a] Postburn Days	Bacteremia[a] Micro-organism	Pneumonia[b] Postburn Days	Pneumonia[b] Micro-organism	Peripheral Alimentation[c] Postburn Days
1[d]	14-16, 30	Staphylococcus aureus	None		7-29
2	10-14, 36	Staphylococcus aureus	25-32	Enterobacter cloacae	13-65
	23	Candida albicans	34-42	Enterobacter cloacae	
	28	enterococcus		Staphylococcus aureus	
3	13	Staphylococcus aureus Staphylococcus epidermidis	None		None
4	15-18, ,44	Staphylococcus aureus	11-18	Hemophilus influenzae	7-16
	33,44	Staphylococcus epidermidis			
	33	diphtheroids			
5	14,32,35,55,56	Staphylococcus aureus	49-60	Staphylococcus aureus	15-62
6	14,15,18,25,29 32,36-39,41,43	Staphylococcus aureus	None		16-66
	11	Staphylococcus epidermidis			
	11	enterococcus			
	36	Klebsiella pneumoniae			
	36	Escherichia coli			

(Continued on next page)

Table 2 (Continued)

Patient No.	Bacteremia[a] Postburn Days	Bacteremia[a] Micro-organism	Pneumonia[b] Postburn Days	Pneumonia[b] Micro-organism	Peripheral Alimentation[c] Postburn Days
7[e]	10	Staphylococcus aureus	4-21	Staphylococcus aureus Proteus mirabilis	None
8[e]	12,14	Staphylococcus aureus	13-44	Staphylococcus aureus Klebsiella pneumoniae Pseudomonas aeruginosa Proteus mirabilis	7-31
9	19	Staphylococcus aureus	None		None
10	25	Staphylococcus aureus	None		None
11[e]	7,33 19 20 20,26,28,41	Staphylococcus aureus diphtheroids Pseudomonas aeruginosa Candida tropicalis	5-20 46-59	Staphylococcus aureus Klebsiella pneumoniae Pseudomonas aeruginosa Pseudomonas aeruginosa Pseudomonas multaphilia	12-18, 20-24
12[e]	27 28-30,35 29	Staphylococcus aureus Candida albicans Pseudomonas aeruginosa	18-69	Enterobacter cloacae Proteus mirabilis Pseudomonas aeruginosa Klebsiella pneumoniae	23-30, 40-43

[a] Bacteremia was documented by positive blood cultures.
[b] Pneumonia was documented by positive sputum cultures, chest x-rays, and clinical findings.
[c] 10% dextrose in water plus 4.5% Free Amine and daily infusions of Intralipid were used.
[d] Patient 1 died of pulmonary edema on day 32.
[e] Patients 7, 8, 11, and 12 died of pneumonia on days 21, 44, 59, and 69 respectively.

added to polypropylene capped tubes, and the tubes were centrifuged at 1000 g for 15 minutes at 4° C. The supernatants were discarded, and 392 µl of the test serum and 8 µl of the bacterial suspension (8.0×10^5 cells) were added to the leukocyte pellets. Controls consisting of serum and bacteria were prepared in tubes lacking leukocytes, and controls consisting of leukocytes and bacteria only were prepared by substitution of HBG for the serum. The tubes were gently vortexed and then rotated at 37° C. Samples (50 µl) were removed at 0 time and after 30 and 60 minutes of incubation. The samples were diluted in distilled water to rupture the leukocytes, and serial 10-fold dilutions were plated on brain heart infusion agar. The plates were incubated at 37° C overnight, and the colonies were enumerated. The percent of bactericidal activity was determined by the formula $\frac{a - b}{a} \times 100$, where a was equal to the number of total surviving bacteria at 0 time and b was equal to the number of total surviving bacteria after 30 or 60 minutes of incubation. The percent of inhibition of PMN bactericidal activity was calculated by the same formula, where a was equal to the percent of PMN bactericidal activity in the presence of pooled normal human serum and b was equal to the percent of PMN bactericidal activity in the presence of the patient's serum.

In some experiments, surviving extracellular bacteria were measured in addition to total surviving bacteria. For these experiments, the volumes of the reactants were doubled and 150 µl samples were removed at each time period. Fifty µl of the sample were used for the enumeration of total surviving bacteria, and the remaining 100 µl were centrifuged at 200 g for 5 minutes at 4° C. Extracellular bacteria in the supernatants were enumerated by plating serial 10-fold dilutions on brain heart infusion agar and colony counting after overnight incubation at 37° C.

In other experiments, the bacteria were opsonized with the test serum prior to incubation with the leukocytes. One ml of the bacterial suspension containing 1.0×10^8 cells was centrifuged at 5000 g for 15 minutes at 4° C. The supernatant was discarded, and 392 µl of the test serum and 8 µl of HBG were added to the bacterial pellet. HBG was substituted for the serum in the control. The bacteria were suspended in the serum or HBG and incubated at 37° C for 30 minutes. The bacteria were washed twice and resuspended in HBG to a final concentration of 1.0×10^8 cells/ml. The opsonized bacteria (8 µl), 4.0×10^6 PMNs, and HBG were added to polypropylene capped tubes in a final volume of 400 µl. The rest of the procedure was identical to that described for enumeration of total surviving bacteria.

In one series of experiments, the methodology described above was further modified. Eight hundred µl of the leukocyte suspension (8.0×10^6 PMNs) were added to polypropylene capped tubes, and the tubes were centrifuged at 1000 g for 15 minutes at 4° C. The supernatants were discarded, and 784 µl of the test serum and 16 µl of HBG were added to the leukocyte pellets. For the preparation of diluent-treated leukocytes, HBG was substituted for the test serum. The leukocytes were suspended in the test serum or HBG and incubated at 37° C for 30 minutes. The

leukocytes were deposited by centrifugation, washed once with 1 ml of HBG, and resuspended in 784 µl of HBG. Sixteen µl of bacteria opsonized with pooled normal human serum ($1.6x10^6$ cells) were added to the leukocytes, and total and extracellular surviving bacteria were enumerated at the various time periods described above.

Viability of the serum-treated PMNs was measured by trypan blue dye exclusion. The leukocytes were treated with the test serum or diluent as described above. Two hundred µl of the leukocyte suspension were mixed with an equal volume of 1% trypan blue dye in physiologic saline, pH 7.0, and incubated for 10 minutes at room temperature. The percent of viable PMNs which excluded the dye in each sample was microscopically estimated by counting 200 PMNs. A positive control consisting of leukocytes which had been heated at 56° C for 1 hour was included in the experiments; the percent of viable PMNs in this control was shown to be 2%.

In our initial experiments, each patient's serial serum samples and pooled normal human serum were tested for their ability to kill that patient's specific infecting S. aureus strain in the presence and absence of normal human PMNs. Killing of the bacteria by the PMNs in the absence of serum was also determined. Neither the patients' sera nor the normal serum facilitated killing of the bacterial strains in the absence of PMNs. Killing of the bacteria by PMNs in the absence of serum was also not demonstrated. In the presence of PMNs and normal serum, killing of the bacterial strains ranged from 87% to 94% after 30 minutes of incubation and from 93% to 99.9% after 60 minutes of incubation. Bactericidal activity at both time periods was decreased in the presence of PMNs and sera from Patients 2, 11, and 12. Bactericidal activity in the presence of PMNs and sera from the other patients was equivalent to bactericidal activity in the presence of PMNs and normal serum.

The temporal sequence of the inhibitory effect of sera from Patients 2, 11, and 12 on the bactericidal activity of normal human PMNs is shown in Figure 1. All of Patient 2's sera, which were obtained after 20 days postburn, markedly inhibited PMN bactericidal activity. Sera from Patients 11 and 12 had no inhibitory effect on PMN bactericidal activity until after the 8th postburn day. In Patient 11, inhibitory activity was demonstrated from the 16th to the 30th postburn day. In Patient 12, inhibitory activity was demonstrated from the 13th to the 43rd postburn day.

The next series of experiments were designed to determine the effects of the burn sera on the opsonization, phagocytosis, and intracellular killing processes. For these experiments, the sera from Patients 2 and 12 were used. A pool of serum was prepared on each patient consisting of equal parts of all sera obtained from Patient 2 and sera obtained from Patient 12 during 13 to 43 days postburn. Patient 11 was not studied, because the amount of sera with inhibitory activity available on this patient was insufficient for use in the experiments.

To determine if the inhibition of PMN bactericidal activity was

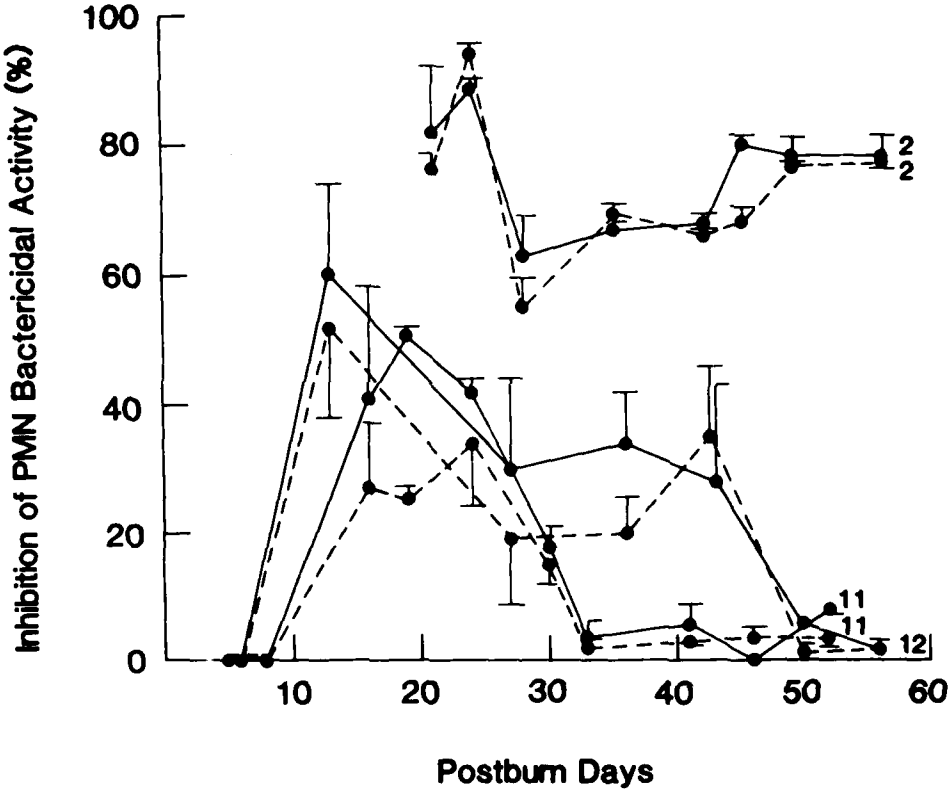

Figure 1. Temporal sequence of the inhibitory effect of sera from Patients 2, 11, and 12 on the bactericidal activity of normal human PMNs. The solid lines represent the percent of inhibition of PMN bactericidal activity after 30 minutes of incubation, and the dotted lines represent the percent of inhibition of PMN bactericidal activity after 60 minutes of incubation. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

caused by deficient opsonization of the bacteria by the burn sera, the following experiments were performed. Washed cells of the infecting S. aureus strains from Patients 2 and 12 were incubated with the respective patient's pooled serum, pooled normal human serum, or diluent. The bacteria were washed, incubated with normal human PMNs, and bactericidal activity was determined. Bactericidal activity was also measured concurrently in reaction mixtures consisting of non-opsonized bacteria, leukocytes, and patients' sera, normal serum, or diluent. Inhibition of PMN bactericidal activity was demonstrated when the serum from each patient was directly incubated with the bacteria and leukocytes (Figure 2). However, when the bacteria were opsonized with the patients' sera, washed, and then incubated with the leukocytes, no inhibition of PMN bactericidal activity was demonstrated. These results indicated that the reduction in PMN bactericidal activity demonstrated in the presence of the patients' sera was not caused by inability of the sera to facilitate opsonization of the bacteria.

In our next experiments, the ability of the burn sera to inhibit the phagocytosis and intracellular killing processes was investigated. Total and extracellular bacterial counts were measured in reaction mixtures consisting of washed cells of the infecting S. aureus strains, normal human PMNs, and patients' sera, pooled normal human serum, or diluent. As demonstrated previously, PMN bactericidal activity was decreased in the presence of the patients' sera in comparison to PMN bactericidal activity in the presence of normal serum (Figure 3). Extracellular bacterial counts were equivalent to total bacterial counts in all reaction mixtures. These results suggested that the burn sera inhibited phagocytosis of the bacteria by the leukocytes rather than intracellular killing.

Our next experiments were performed to determine if the inhibition of phagocytosis was related to a toxic effect of the burn sera on the leukocytes. Normal human PMNs were incubated with each patient's pooled serum, pooled normal human serum, or diluent. The leukocytes were washed once, and viability was measured by trypan blue dye exclusion. No decrease in viability was demonstrated after incubation of the leukocytes with the sera, indicating that the inhibitory effect of the burn sera on the phagocytic process was not associated with cell death.

In our next experiments, the ability of the burn sera to inhibit phagocytosis of E. coli 075 by normal human PMNs was determined. Total and extracellular bacterial counts were measured in reaction mixtures consisting of washed cells of the test strain, normal human PMNs, and patients' sera, pooled normal human serum, or diluent. The number of total and extracellular bacteria surviving after incubation with the patients' sera and leukocytes was increased in comparison to the number surviving after incubation with the normal serum and leukocytes (Figure 4). These results indicated that the burn sera inhibited phagocytosis of E. coli as well as S. aureus.

Our final experiments were designed to investigate the mechanism by which the burn sera inhibited phagocytosis. Normal human PMNs were

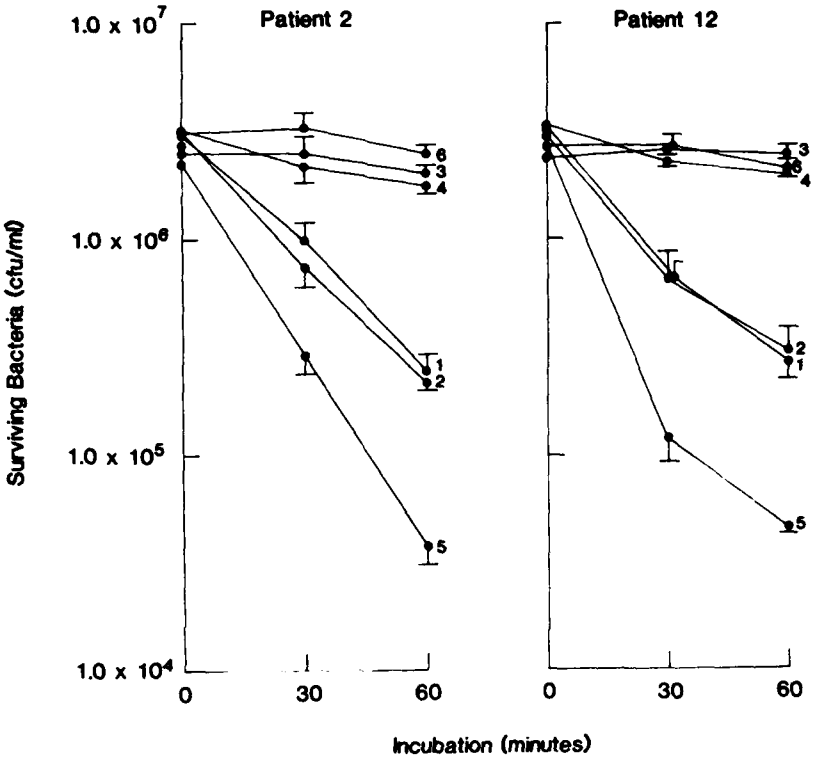

Figure 2. Intracellular killing by normal human PMNs of S. aureus opsonized with burn sera or normal serum. The results of the experiments performed with the pooled serum from Patient 2 are presented in the left figure, and the results of the experiments performed with the pooled serum from Patient 12 are presented in the right figure. All reaction mixtures consisted of leukocytes and the following reactants: (1) bacteria opsonized with patient's serum; (2) bacteria opsonized with pooled normal human serum; (3) bacteria opsonized with diluent; (4) non-opsonized bacteria and patient's serum; (5) non-opsonized bacteria and pooled normal human serum; and (6) non-opsonized bacteria. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

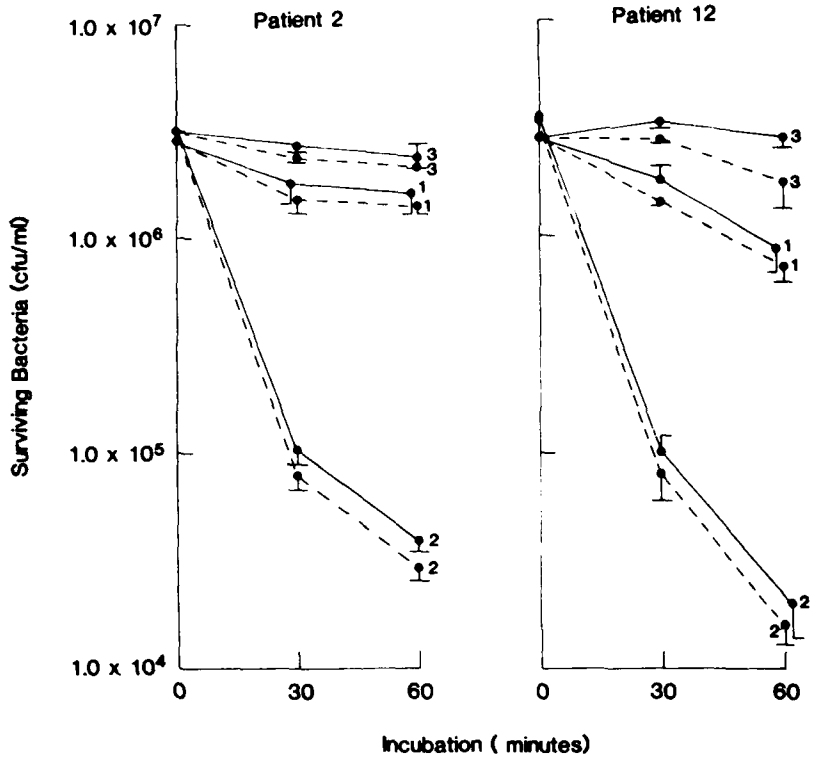

Figure 3. Phagocytosis and intracellular killing by normal human PMNs of S. aureus in the presence of burn sera or normal serum. The results of the experiments performed with the pooled serum from Patient 2 are presented in the left figure, and the results of the experiments performed with the pooled serum from Patient 12 are presented in the right figure. All reaction mixtures consisted of bacteria, leukocytes, and the following reactant: (1) patient's serum, (2) pooled normal human serum, and (3) diluent. The solid lines represent total surviving bacteria, and the dotted lines represent surviving extracellular bacteria. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

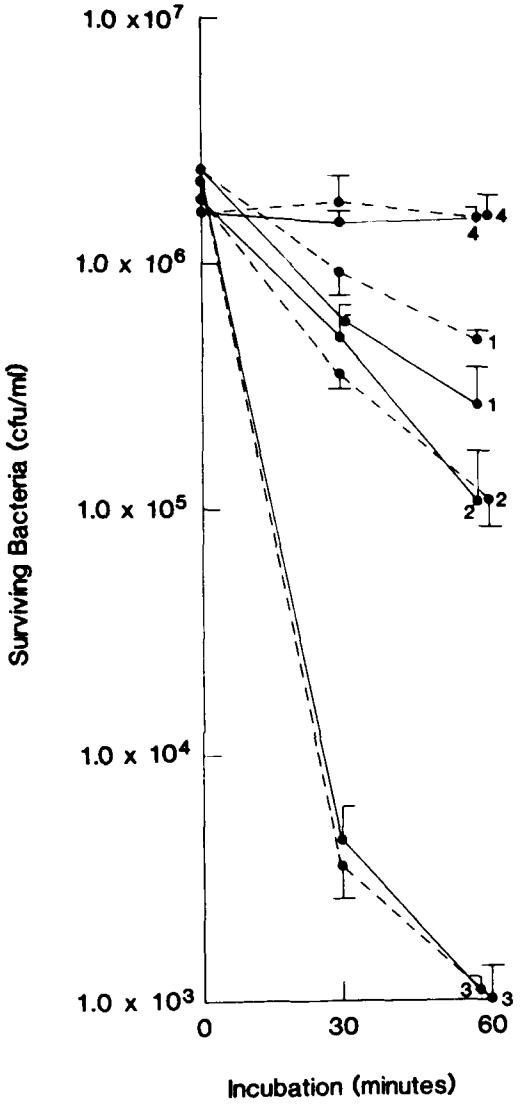

Figure 4. Phagocytosis and intracellular killing by normal human PMNs of E. coli 075 in the presence of burn sera or normal serum. All reaction mixtures consisted of bacteria, leukocytes, and the following reactant: (1) Patient 2's serum, (2) Patient 12's serum, (3) pooled normal human serum, and (4) diluent. The solid lines represent total surviving bacteria, and the dotted lines represent surviving extracellular bacteria. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

incubated with each patient's pooled serum, pooled normal human serum, or diluent. The leukocytes were washed once and then tested for their ability to phagocytose washed cells of the infecting S. aureus strains opsonized with pooled normal human serum. The number of total and extracellular surviving bacteria was increased after incubation with the burn serum-treated leukocytes in comparison to the number surviving after incubation with the normal serum or diluent-treated leukocytes (Figure 5). These results showed that the inhibitory effect of the burn sera on the phagocytic process resulted from a direct interaction of the sera with the leukocytes, which was not reversed by washing of the leukocytes.

2. Discussion

The results of the present investigation show that the sera from 3 of 12 burned patients inhibited the bactericidal activity of normal human PMNs when tested at a physiologic concentration. In the 2 patients whose sera were available for testing during the first 2 weeks postburn, inhibitory activity was not demonstrated until after the 8th postburn day. Inhibitory activity persisted in one of the patients until the 32nd postburn day and, in the other, until the 50th day. In the third patient, who was not enrolled on the study until the 21st postburn day and was followed until the 56th day, inhibitory activity was demonstrated in all sera obtained during that time.

PMN bactericidal activity involves a complex series of events including opsonization, phagocytosis, and intracellular killing. The ability of sera from 2 of the 3 patients to inhibit each of these processes was investigated. The patients' sera were first tested at a physiologic concentration for their ability to support opsonization of the bacteria. No diminution in opsonization by the burn sera was demonstrated, indicating that decreased PMN bactericidal activity was not caused by an inhibitory effect of the sera on the opsonization process. To determine if the burn sera inhibited the phagocytosis or intracellular killing process, the number of extracellular bacteria remaining in the supernatants after incubation of the bacteria with the leukocytes and sera was compared to the number of total surviving bacteria (extracellular and intracellular). No significant difference in the bacterial counts was observed, indicating that all of the bacteria which were phagocytosed were killed intracellularly. However, the number of extracellular bacteria remaining in the supernatants after incubation of the bacteria with leukocytes and burn sera was markedly increased in comparison to the number of extracellular bacteria remaining in the supernatants after incubation of the bacteria with leukocytes and normal serum. These results suggested that the burn sera inhibited the phagocytic process and thereby reduced the number of bacteria available for intracellular killing.

The results described above were obtained from experiments in which each patient's infecting S. aureus strain was exclusively used. The ability of the patients' sera to inhibit phagocytosis of an E. coli strain by normal human PMNs was also investigated, and an inhibitory

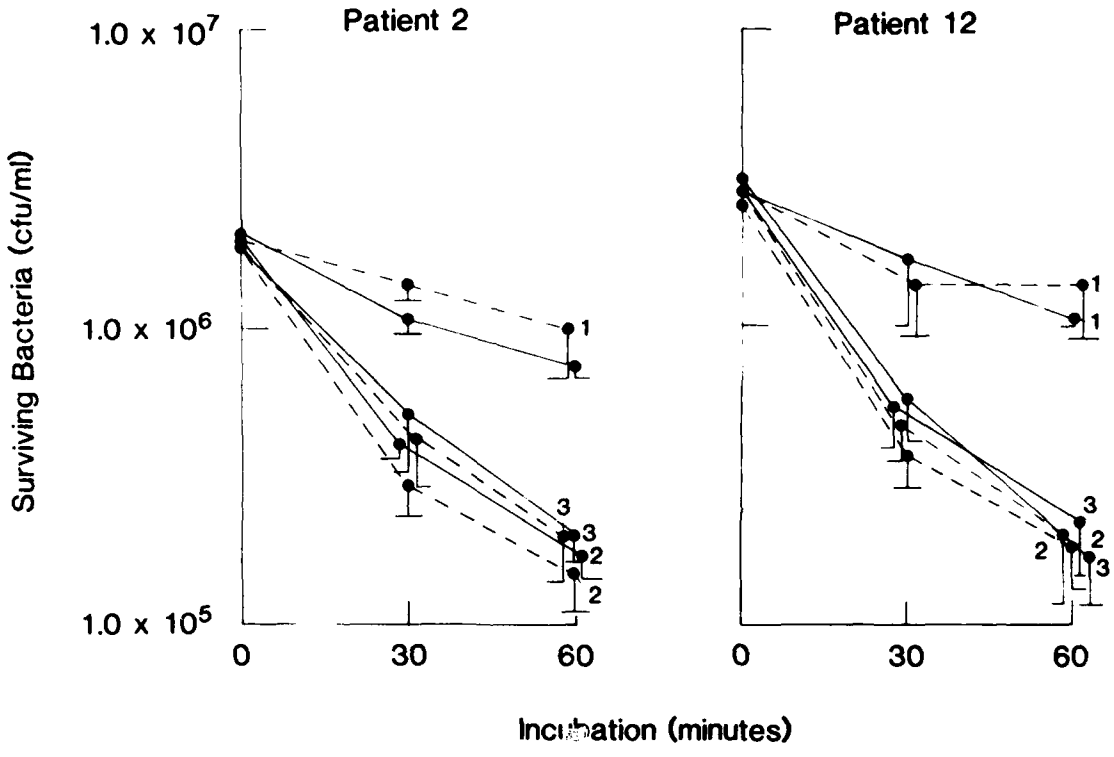

Figure 5. Phagocytosis and intracellular killing of S. aureus opsonized with pooled normal human serum by serum-treated normal human PMNs. The results of the experiments performed with the pooled serum from Patient 2 are presented in the left figure, and the results of the experiments performed with the pooled serum from Patient 12 are presented in the right figure. The reaction mixtures consisted of the following reactants: (1) leukocytes treated with patient's serum and bacteria opsonized with normal serum; (2) leukocytes treated with normal serum and bacteria opsonized with normal serum; and (3) leukocytes treated with diluent and bacteria opsonized with normal serum. The solid lines represent total surviving bacteria,and the dotted lines represent surviving extracellular bacteria. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

effect was demonstrated. The observation that the burn sera inhibited phagocytosis of both S. aureus and E. coli suggests that the inhibitory effect was not dependent on bacterial surface properties.

The mechanism by which the burn sera inhibited phagocytosis was also investigated. Normal human PMNs were incubated with the burn sera, washed, and then tested for viability. The leukocytes remained fully viable, indicating that the inhibitory effect on phagocytosis was not associated with cell death. The burn serum-treated PMNs were also tested for their ability to phagocytose and kill S. aureus opsonized with normal human serum. Inhibition of phagocytosis of the opsonized bacteria by the burn serum-treated PMNs was demonstrated. Thus, the inhibitory effect resulted from a direct interaction of the burn sera with the leukocytes, which was not reversed by washing of the leukocytes. The burn serum inhibitor may have exerted its effect by binding avidly to the membrane of the leukocytes. Alternatively, the inhibitor may have been pinocytosed and adversely affected the contractile elements within the leukocytes.

The observation that the inhibitory effect of the burn sera was not rapidly reversible suggests that it may be responsible for abnormalities of PMN function which have been previously demonstrated in burned patients, i.e., decreased phagocytosis (43) and chemotaxis (44-48). These acquired abnormalities have not been generally thought to be mediated by serum inhibitors, because they were demonstrated utilizing normal human serum as the source of opsonins or chemotactic factors. However, if the leukocytes were affected by an inhibitor in circulation and the effect was not reversed by washing, then abnormal leukocyte function could be demonstrable regardless of the source of opsonins or chemotactic factors.

The identity of the burn serum inhibitor of PMN function demonstrated in our investigation is unknown. The inhibitor was retained after dialysis using 12,000 molecular weight retention dialysis tubing, suggesting that its molecular weight is greater than 12,000 daltons. The inhibitor appears to be distinct from the burn serum inhibitors of alternative complement pathway activity (4,5) and T-lymphocyte mediated responses (3,49-54), because these latter inhibitors are of lower molecular weight (50 and sections II B and IV B of this report).

Knowledge of the cause of the occurrence of the burn serum inhibitor of PMN phagocytosis should aid in determining its identity. Comparison of the clinical characteristics of the patients whose sera contained inhibitory activity to those of the patients whose sera lacked inhibitory activity only revealed that the occurrence of the activity was not related to total burn size nor to the amount of third degree burn injury. The 3 patients whose sera contained inhibitory activity had pneumonia, candidemia, and multiple episodes of bacteremia caused by more than one microorganism, and 2 had a fatal outcome. The 3 patients also were receiving peripheral alimentation during the time when the inhibitory activity was demonstrated. However, of the 8 patients whose sera lacked inhibitory activity, 5 received peripheral alimentation, 4 had pneumonia

associated with bacteremia, and 3 had a fatal outcome. A careful review of the types and amounts of blood products administered to the 2 groups of patients did not reveal notable differences. Review of medications also did not provide a clue regarding the cause of the inhibitory activity. The 3 patients with inhibitory activity received amphotericin B for treatment of candidemia; however, therapy with this drug was initiated after inhibitory activity was demonstrated.

The cause and significance of the serum-mediated inhibition of PMN phagocytosis remains to be determined. It is the opinion of the investigators that this information can best be derived from studies using a burned animal model. The advantage of its use is that most of the variables described above can be adequately controlled.

B. Studies to further Investigate the Reduction in Alternative Complement Pathway Mediated C3 Conversion in Burned Patients.

1. Results

Eighteen patients ranging in age from 3 to 81 years were studied. The group included 12 males and 6 females. All patients had flame burn injuries, and the total burn sizes ranged from 20% to 80%. The patients were divided into 3 groups according to the amount of third degree burn injury. Group A consisted of 4 patients with primarily partial thickness injuries with isolated areas of third degree injury. Group B consisted of 8 patients with 10% to 22% third degree injury. Group C consisted of 6 patients with 30% to 75% third degree injury. The ages, sex, and specific burn sizes of the patients within each group are presented in Table 3.

Clotted blood specimens were obtained from the patients upon admission and then 1 or 2 times weekly for up to 50 days postburn. The blood specimens were allowed to clot for 1 hour at room temperature and were centrifuged at 5,000 g for 15 minutes at 4° C. The sera were removed, divided into small aliquots, and frozen at -70° C. Normal human sera were collected from 25 healthy adult donors and stored individually or as a pool at -70° C.

C3 conversion was measured by the method of Ruley et al. (55). Antiserum to human C3 was raised in goats by multiple subcutaneous injections of purified human C3 in physiologic saline, pH 7.0, containing 0.01 M EDTA. Antiserum to the B antigenic determinant of C3 was prepared by adsorbing the antiserum to C3 with the minimal amount of aged normal human serum which completely removed antibodies to the A and D antigenic determinants (56). Radial immunodiffusion using an appropriate dilution of the antiserum was carried out in 1% agarose in veronal buffer (μ = 0.05,pH = 8.6) containing 0.04 M EDTA at room temperature for 18 hours using 2 mm diameter wells. The activating substances which were used for the measurement of C3 conversion were inulin and CoVF, and the initial concentrations of the activating substances were 100 mg/ml and 100 units/ml respectively. The activating substances were added to sera in the

Table 3. Ages, Sex, Burn Sizes and Grouping of the Patients.

Patient No.	Age	Sex[a]	Body Surface Injured (%)[b] Total	Third Degree	Group
1	22	M	20	<10	A
2	25	M	31	<10	
3	31	M	33	<10	
4	57	M	45	<10	
5	49	M	40	10	B
6	38	F	28	10	
7	49	M	33	10	
8	12	M	52	12	
9	81	F	24	14	
10	42	M	41	17	
11	77	M	23	22	
12	46	F	23	22	
13	3	F	40	30	C
14	11	F	40	37	
15	16	F	51	46	
16	21	M	51	47	
17	10	M	58	51	
18	6	M	80	75	

a M = male; F = female.

b All patients had flame burn injuries.

proportion of 10 µl of activating substance to 100 µl of serum, and the mixtures were incubated for 1 hour at 37° C. Samples were removed at 0 time and after 60 minutes of incubation, and the concentration of B antigen of C3 in the samples was quantitated by radial immunodiffusion. The results were expressed in percent of C3 conversion and were calculated by the formula $\frac{a - b}{a} \times 100$, where a was equal to the concentration of B antigen at 0 time and b was equal to the concentration of B antigen after 60 minutes of incubation.

In some experiments, fractions of dialysates of burn sera were tested for their ability to inhibit C3 conversion in burn serum or pooled normal human serum. For these experiments, the reaction mixtures consisted of 75 ul of burn serum or normal serum, 25 ul of the respective fraction, and 10 ul of the activating substance. Physiologic saline, pH 7.0, was substituted for the fraction in the controls. C3 conversion in the reaction mixtures and controls was measured by radial immunodiffusion as described above. The results were expressed as percent of inhibition of C3 conversion by the formula $\frac{a - b}{a} \times 100$ where a was equal to the percent of C3 conversion in the control, and b was equal to the percent of C3 conversion in the reaction mixture.

In our initial experiments, C3 conversion by inulin and CoVF was measured in the patients' serial serum samples and in the normal human sera. The data were grouped and subjected to statistical analysis to determine the differences in C3 conversion between the 3 patient groups and between each patient group and the control. Statistical analysis was performed by Student's t test.

C3 conversion by inulin and CoVF in the sera of all 3 patient groups was significantly decreased during 50 days postburn in comparison to C3 conversion in the normal sera ($p = <0.05$) (Figure 6). The differences between the data from the patient groups and controls were most significant when CoVF was used as the activating substance ($p = <0.01$). The most marked reduction in C3 conversion by both activating substances occurred in group C. Although C3 conversion in group B was greater than in group C, a significant difference between the data from these 2 groups was not demonstrated. The least reduction in C3 conversion occurred in group A, and the differences between the data from groups A and C during 40 days postburn were found to be significant ($p = <0.05$). When the patients were regrouped according to total burn sizes of 20% to 28%, 31% to 40%, and 41% to 80%, again the data between the patient groups and controls were found to be significant ($p = <0.05$). However, significant differences between the 3 groups were not demonstrated. These results suggested that the extent of the reduction in C3 conversion was related to the amount of full thickness injury rather than to the total burn size.

All of the patients in groups B and C, except Patients 5 and 12, had one or more episodes of bacteremia and half of these patients also had bacterial pneumonia during the study period. Therefore, an attempt was made to investigate the relationship between the reduction in C3 conversion and infectious complications by comparing the data from the

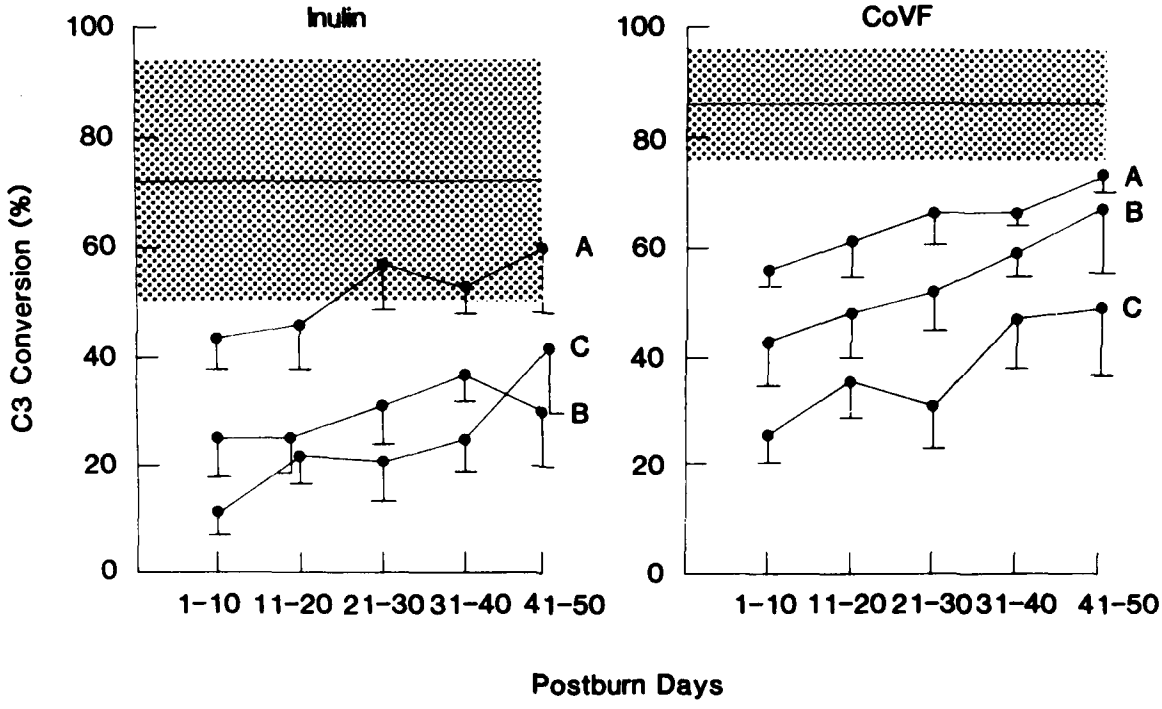

Figure 6. C3 conversion in the sera of patients in Groups A, B, and C. The results obtained using inulin as the activating substance are shown in the left figure, and the results obtained using CoVF as the activating substance are shown in the right figure. The shaded areas represent the range of values (mean $\pm$ 2 SD) obtained from determinations on 25 normal human sera. The points represent mean values of the determinations on the patients in each group at the various time intervals, and each vertical bar represents the standard error of the mean.

patients with bacteremia only to the data from the patients with bacteremia and pneumonia. The group of patients with bacteremia only included Patients 6, 8, 13, 14, 15, and 18. The group of patients with bacteremia and pneumonia included Patients 7, 9, 10, 11, 16, and 17. Significant differences between the data from these 2 patient groups at the various time periods were not demonstrated (Figure 7). These results suggested that the extent of the reduction in C3 conversion was not related to the type of infectious complication.

To determine the relationship between the reduction in C3 conversion and survival after infectious complications, the data from surviving and non-surviving patients with infectious complications were compared. Since all of the patients, except one, survived until the 30th postburn day, the comparisons were made on data obtained during that time. The group of surviving patients included Patients 6, 8, 13, 14, 15, and 16. The non-surviving patients included Patients 7, 9, 10, 11, 17, and 18. Significant differences between the data from these 2 patient groups were not demonstrated at any of the time periods (Figure 8). These results suggested that the extent of the reduction in C3 conversion was not predictive of fatal outcome resulting from infectious complications.

In our next experiments, the ability of dialysis to correct the reduction in C3 conversion in the burn sera was investigated. Individual sera with reduced C3 conversion obtained during 30 days postburn from Patients 6, 12, 13, 14, 16, 17, and 18 were pooled, and each patient's pooled serum was dialyzed for 18 hours at 4° C against PBS^{2+} using 3,500 or 12,000 molecular weight retention dialysis tubing or incubated for 18 hours at 4° C without dialysis. C3 conversion by inulin and CoVF was measured in these sera and compared to C3 conversion in similarly treated pooled normal human serum and untreated burn sera. Dialysis of the burn sera using either the 3,500 or 12,000 molecular weight retention dialysis tubing partially or, in one case, fully corrected the reduction in C3 conversion by inulin (Table 4). C3 conversion by inulin in burn sera which had been incubated at 4° C without dialysis was identical to C3 conversion by inulin in untreated burn sera. C3 conversion by CoVF in the dialyzed burn sera was also increased in comparison to C3 conversion by CoVF in burn sera which had been incubated at 4° C without dialysis or in untreated burn sera (Table 5). However, the amount of correction of the reduction in C3 conversion after dialysis using CoVF as the activating substance was less than that observed when inulin was used as the activating substance. Magnesium concentrations in the untreated burn sera were within the normal range (1.8 to 2.6 mg/dl), suggesting that the correction in C3 conversion by dialysis was not related to normalization of the concentration of magnesium ions.

Our next experiments were performed to determine if an inhibitory activity could be isolated from the dialysates of the burn sera. Five hundred µl of each pooled burn serum or pooled normal human serum were dialyzed for 18 hours at 4° C against 50 ml of PBS^{2+} using 3,500 molecular weight retention dialysis tubing. The dialysates were lyophilized

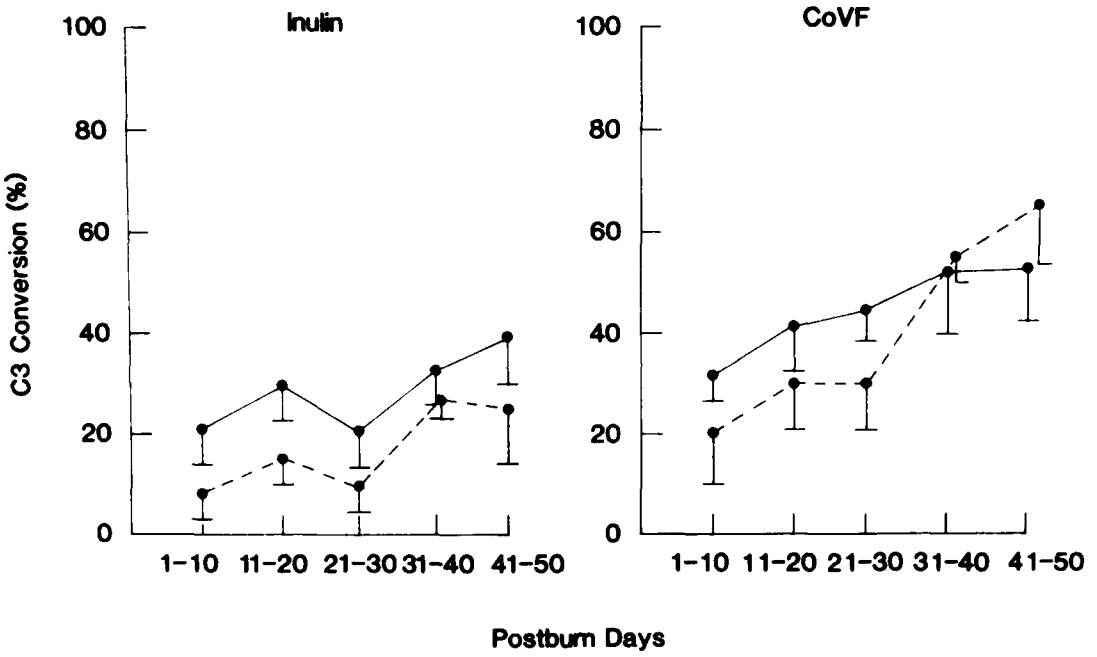

Figure 7. C3 conversion in the sera of pateints with bacteremia only and of patients with bacteremia and pneumonia. The results obtained using inulin as the activating substance are shown in the left figure, and the results obtained using CoVF as the activating substance are shown in the right figure. The solid line represents the data on the patients with bacteremia only, and the dotted line represents the data on the patients with bacteremia and pneumonia. The points represent mean values of the determinations on the patients in each group at the various time intervals, and each vertical bar represents the standard error of the mean.

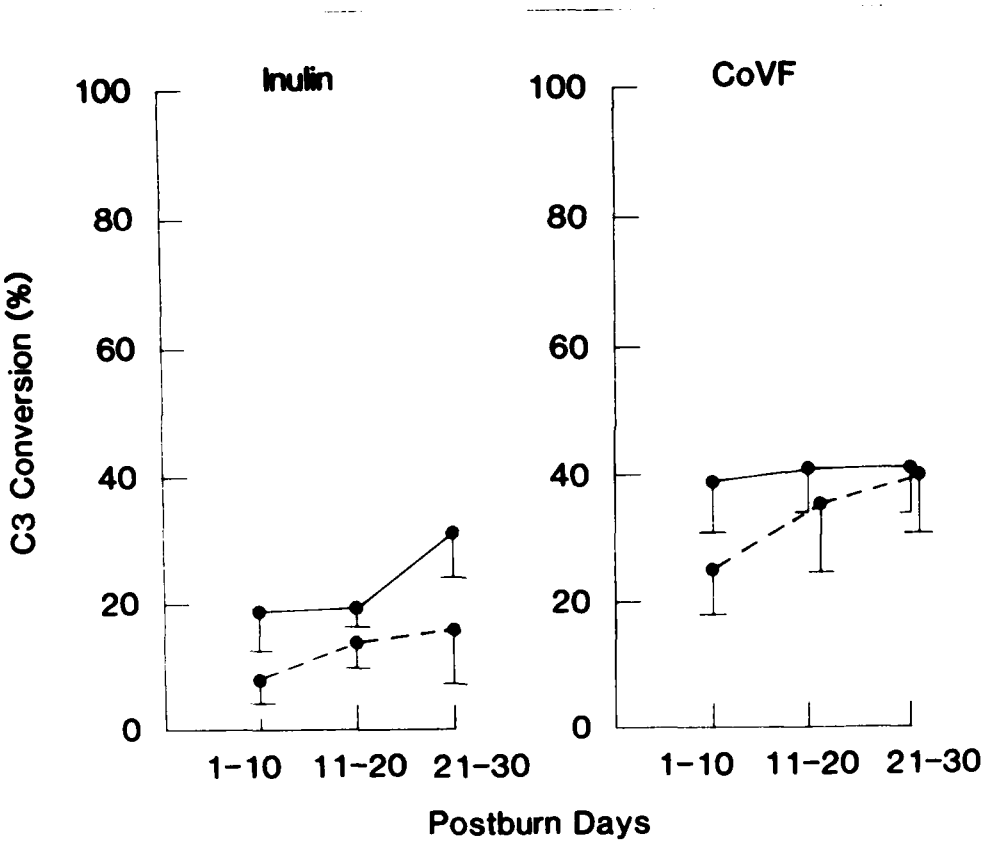

Figure 8. C3 conversion in the sera of surviving and non-surviving patients with infectious complications. The results obtained using inulin as the activating substance are shown in the left figure, and the results obtained using CoVF as the activating substance are shown in the right figure. The solid line represents the data on the surviving patients, and the dotted line represents the data on the non-surviving patients. The points represent mean values of the determinations on the patients in each group at the various time intervals, and each vertical bar represents the standard error of the mean.

Table 4. Effect of Dialysis on C3 Conversion by Inulin in Burn Sera.

Patient No.	C3 Conversion (%)[a]			
	Untreated	After Incubation[b]	After Dialysis[c] 3,500	12,000
6	37.3	41.8	57.0	65.4
12	33.9	34.2	49.2	52.0
13	34.0	34.0	62.4	67.6
14	29.2	30.5	84.9	86.4
16	14.2	12.4	42.3	64.0
17	26.9	31.4	50.2	66.4
18	8.4	11.0	33.6	46.4
PNHS[d]	71.0	71.5	75.4	73.2

[a] Numbers represent mean values of 2 to 6 determinations.

[b] Sera were incubated at 4°C for 18 hours without dialysis.

[c] Sera were dialyzed at 4°C for 18 hours against PBS^{2+} using 3,500 or 12,000 molecular weight retention dialysis tubing.

[d] PNHS = pooled normal human serum.

Table 5. Effect of Dialysis on C3 Conversion by CoVF in Burn Sera.

Patient No.	C3 Conversion (%)[a]			
	Untreated	After Incubation[b]	After Dialysis[c] 3,500	12,000
6	62.0	61.9	70.1	69.4
12	61.0	64.0	72.8	75.9
13	46.5	49.1	69.4	68.2
14	40.3	43.2	59.2	62.4
16	37.9	38.3	51.5	50.3
17	37.7	35.1	48.5	57.6
18	36.9	37.4	49.8	53.9
PNHS[d]	84.2	85.0	82.1	84.1

[a] Numbers represent mean values of 2 to 6 determinations.

[b] Sera were incubated at 4°C for 18 hours without dialysis.

[c] Sera were dialyzed at 4°C for 18 hours against PBS^{2+} using 3,500 or 12,000 molecular weight retention dialysis tubing.

[d] PNHS = pooled normal human serum.

and then redissolved in 25 ml of distilled water. Each dialysate was chromatographed on a 2.6x25 cm column of Sephadex G-25. Five ml fractions were collected, and protein in the fractions was detected spectrophotometrically at 280 nm. Fractions of each dialysate containing protein (#19 to #37) were lyophilized, redissolved in 500 ul of physiologic saline, pH 7.0, and tested for their ability to inhibit C3 conversion by inulin and CoVF in pooled normal human serum and in the dialyzed sera from which each had been prepared. None of the fractions of the dialysates inhibited C3 conversion in the sera. These results suggested either that the fractions containing protein did not contain inhibitory activity or the inhibitor was present in the fractions in too low a concentration to be detectable by the methods employed.

2. Discussion

The complement system is the primary humoral mediator of biological events associated with host resistance against microbial infection, i.e., changes in vascular permeability, leukocyte chemotaxis, opsonization leading to phagocytosis of microorganisms, bacteriolytic activity, and neutralization of viruses (57). The alternative pathway of complement activation is that part of the complement system which provides the non-immune host with natural defense against microbial infection. The alternative pathway also functions in the immune host by amplifying activation of the classical complement pathway.

The six serum proteins which constitute the alternative complement pathway are factor B (B), factor D ($\overline{D}$), properdin (P), C3, C3bINA, and β1H (58-61). Two C3 convertases are formed in the reaction sequence of the alternative pathway (Figure 9). The first convertase, $\overline{C3Bb}$, is thought to be assembled in the fluid phase during the slow interaction of B, $\overline{D}$, and C3 in the presence of magnesium ions, and it continuously provides small amounts of C3b that can initiate formation of the amplification convertase, $\overline{C3bBb}$ (59,61). B binds to C3b in the presence of magnesium ions and is cleaved by $\overline{D}$ to uncover the C3-cleaving site on the Bb fragment of B (62). $\overline{C3bBb}$ rapidly loses activity by spontaneous dissociation of the Bb fragment, which becomes inactive Bbi, and P serves to stabilize convertase activity by binding to the C3b subunit of the enzyme and retarding dissociation of Bb (63). Binding of a second molecule of C3b to the stable or unstable C3 convertase converts the enzyme to a C5 convertase, leading to activation of terminal components of the classical complement pathway (63,64).

The primary role of the regulatory proteins, β1H and C3bINA, is to prevent amplification of C3 convertase formation and activity (65,66). β1H has three inhibitory effects as follows: (a) It competes with B for binding to C3b and thereby blocks $\overline{C3bBb}$ formation; (b) it dissociates Bb from $\overline{C3bBb}$ and thereby inhibits convertase activity; and (c) it increases the susceptibility of C3b to cleavage by C3bINA, thereby yielding C3bi, an inactive form of this protein.

C3b produced by cleavage of C3 by $\overline{C3Bb}$ in the fluid phase binds covalently to particulate material (67). Whether the particulate material

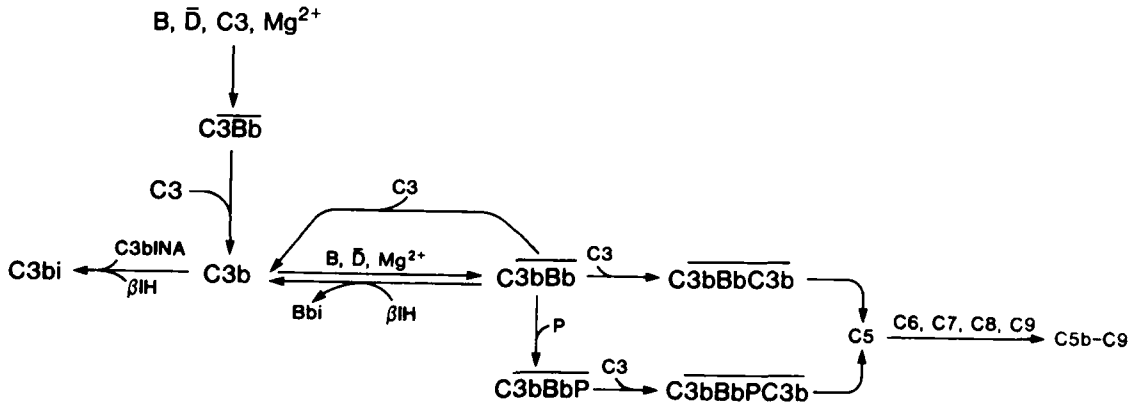

Figure 9. Reaction sequence of the alternative complement pathway.

is an activator or non-activator of the alternative pathway is determined by the relative capacity of its surface to protect C3b from the action of the regulatory proteins, β1H and C3bINA (58,59,61,68-71). Activating particles circumvent the action of the regulatory proteins allowing for formation of $\overline{C3bBb}$ resulting in further deposition of C3b.

In the present investigation, inulin and CoVF were used as the activating substances for the measurement of alternative complement pathway activity in the sera of thermally injured patients. Inulin is a recognized particulate activator of the alternative pathway (72), and CoVF is an analog of C3b (73) which forms a C3 convertase in the fluid phase by interacting with B and $\overline{D}$ in the presence of magnesium ions (74,75).

Reduction in C3 conversion by both activating substances was demonstrated in the sera of 18 burned patients. The most marked reduction in C3 conversion occurred in patients with large full-thickness injuries, who had infectious complications during their clinical course. The abnormality was not, however, more severe in patients with pneumonia and bacteremia in comparison to patients with bacteremia only and was not predictive of fatal outcome resulting from infectious complications.

The demonstration of an increase in the severity of the abnormality as the amount of full-thickness injury increased suggests that its occurrence may be directly or indirectly related to metabolic or physiologic alterations that occur following injury. This concept is further supported by the observation that reduction in C3 conversion utilizing CoVF as the activating substance was also demonstrated in the sera of patients who had sustained severe blunt or penetrating abdominal trauma (76). The abnormality was demonstrated in these patients within 6 hours following trauma, which is the time when metabolic and physiologic alterations are pronounced. The abnormality in the burned patient may increase with infectious complications, because metabolic or physiologic alterations are augmented by tissue pyrogens, endotoxin, or other bacterial constituents or products.

Dialysis of the burn sera was shown to correct the reduction in C3 conversion. The effect was not related to normalization of the concentration of magnesium ions, since magnesium concentrations in non-dialyzed burn sera were found to be normal. It is postulated that the dialysis procedure corrected the reduction in C3 conversion by removing a low molecular weight inhibitor from the burn sera. Our failure to demonstrate inhibitory activity in protein-containing fractions of burn sera dialysates does not rule out this hypothesis, because the methods used may not have been sensitive enough to detect inhibitory activity in the fractions or the inhibitor may not be a protein.

It is our hypothesis that the inhibitor exerts its effect by potentiating β1H activity. C3b in the fluid phase binds β1H with almost 100-fold greater affinity than that with which it binds B, whereas C3b on the surface of a particulate activator, such as inulin, binds β1H less effectively and uptake of this control protein is not favored

relative to uptake of B (59,61,69). Therefore, if our inhibitor augments β1H activity, it would be expected to potentiate the inactivation of the fluid phase analog of C3b, CoVF, and thereby inhibit the formation of the CoVF-dependent C3 convertase more readily than formation of the inulin-dependent C3 convertase. This would explain why the greatest reduction in C3 conversion in the burn sera was demonstrated when CoVF was used as the activating substance and why dialysis of the burn sera corrected the reduction in C3 conversion by inulin more than by CoVF. In addition, it would provide an explanation for the observation that CoVF was able to detect a reduction in C3 conversion in the sera of patients with non-burn trauma, whereas inulin was not (76).

A serum inhibitor has been shown to contribute to abnormal T-lymphocyte blastogenesis associated with burn injury (77) and major operative trauma (78). The immunosuppressive factor is a polypeptide with a molecular weight of less than 10,000 daltons. The observation in our investigation that the inhibitor of alternative complement pathway mediated C3 conversion in burn sera was dialyzable and of low molecular weight suggests that it may be related to or may in fact be the immunosuppressive factor. This concept would not be surprising as evidence accumulates regarding a role for complement in T-lymphocyte dependent responses (79-83).

C. Studies to Determine the Association Between Colonization of Body Surfaces with Candida, the Presence of Candida Antigenemia, and the Occurrence of Systemic Candidosis.

1. Results

The association between colonization of the burn wound, mouth, rectum, and catheter insertion site by Candida and the occurrence of systemic candidosis was studied in 8 severely burned patients receiving TPN therapy. The ages, sex, and burn sizes of these patients are presented in Table 6. Fifteen catheters were inserted in the 8 patients. Of the 15 catheters inserted, 5 were excluded from our study because the catheter tips were not cultured upon termination of TPN therapy. Five catheters were threaded into the superior vena cava by the infraclavicular approach to the subclavian vein in the 4 patients, who were not burned in this area (Table 7). The 4 patients, who were burned over the entire trunk and neck, were treated with 5 catheters inserted through burn eschar; 3 catheters were inserted into the superior vena cava by the infraclavicular approach to the subclavian vein, and 2 catheters were inserted through the cephalic vein into the superior vena cava. Each of the 10 catheters used in the study was in place for an average of 6.9 days (range, 3-14 days). Six of the 10 catheters were silastic, and the remaining four were polyvinyl chloride. Five of the patients were treated with a standard central TPN formulation; 2 patients were treated with renal TPN formulation due to renal failure, and 1 patient received cardiac TPN formulation due to cardiac failure. The components of the central, renal, and cardiac TPN formulations are listed in Table 8.

The central venous catheters used for TPN therapy were inserted under sterile conditions and monitored daily by a member of the University Hospitals' Hyperalimentation Unit. When the catheter was to be placed through intact skin, the insertion site was first disinfected by standard techniques (84). Following catheter insertion, povidone-iodine was placed over the site, and the site was covered with an air-occlusive dressing. The dressings were changed every 48 hours with the use of the mask-and-glove technique. At the time of dressing change, the skin was prepared with acetone, povidone-iodine, and isopropyl alcohol (70%), and povidone-iodine ointment was reapplied to the insertion site. When the catheter was to be inserted through burn eschar, the burn wound at the insertion site was disinfected with povidone-iodine prior to catheter placement. After the catheter was in place, povidine-iodine ointment was applied to the site, and the site was covered with a loose sterile gauze pad. The povidone-iodine ointment at the catheter insertion site was removed every 8 hours with sterile cotton-tipped applicators, and fresh povidone-iodine was applied.

Strict precautions were employed to reduce the potential for exogenous contamination of the catheter, infusion apparatus, and nutrient solution. The infusion catheter was used exclusively for the administration of the hyperalimentation solution; measurements of central venous pressure or the administration of blood products, antibiotics, or

Table 6. Ages, Sex, and Burn Sizes of the Patients.

Patient No.	Age	Sex[a]	Body Surface Injured (%)[b] Total	Third Degree
1	49	M	33	10
2	22	M	62	18
3	72	F	30	25
4	77	M	23	22
5	78	M	18	3
6	42	M	64	59
7	21	M	51	47
8	81	F	24	14

[a] M = male; F = female.

[b] All patients had flame burn injuries.

Table 7. Characteristics of the Methodology Employed for the Administration of TPN Therapy in the Patients.

Patient No.	Days of TPN Therapy	TPN Formulation Administered	Type of Catheter[a]	Insertion Site[b]
1				
Catheter I	8	Central	Sil	R-SVC-NB
Catheter II	14	Central	Sil	L-SVC-NB
2	5	Central	Sil	R-SVC-B
3	3	Cardiac	Sil	L-SVC-NB
4	9	Central	Sil	R-SVC-NB
5	9	Renal	PVC	R-SVC-NB
6	6	Renal	PVC	L-SVC-B
7				
Catheter I	4	Central	PVC	L-Cephalic-B
Catheter II	5	Central	PVC	R-Cephalic-B
8	6	Central	Sil	R-SVC-B

[a] Sil = silastic; PVC = polyvinyl chloride.

[b] R = right; L = left; SVC = subclavian vein; cephalic = cephalic vein; NB = catheter inserted through non-burned skin; B = catheter inserted through burn wound.

Table 8. Composition of the TPN Formulations Administered to the Patients.

Constituents	Central Formulation[a]	Renal Formulation[b]	Cardiac Formulation[c]
Amino acids[d] (gm)	42.5	0	30.6
Amino acids (essential)[e] (gm)	0	12.75	0
Dextrose (gm)	250	350	350
Potassium (meq)	12	0	0
Sodium (meq)	21	1.2	3.6
Magnesium (meq)	8	0	8
Calcium (meq)	4.7	0	0
Chloride (meq)	8	0	0
Acetate (meq)	29	0	16
Phosphorus (mM)	13.4	0	3.6
Total calories (Kcal)	1,000	1,250	1,320
Nitrogen (gm)	6.25	1.46	4.5

[a] Components contained in 1000 ml of formulation.
[b] Components contained in 750 ml of formulation.
[c] Components contained in 860 ml of formulation.
[d] Free Amine (McGaw Laboratories, Glendale, Calif.).
[e] Nephramine (McGaw Laboratories).

other medications through the infusion catheter was specifically prohibited. Intravenous tubing and 0.45 μ filters were changed daily. The nutrient solutions were prepared daily by the hospital pharmacy in a clean room under a laminar-flow hood.

Surface quantitative cultures of a representative area of the skin at the site of insertion of the central venous catheter and of a representative area of each patient's burn wound were performed at the time of catheter insertion. The numbers of bacteria and yeasts in these cultures were quantitated, and all microorganisms isolated were identified. Cultures of the rectum and mouth were also obtained at the time of catheter insertion. Mouth cultures were substituted for cultures of the oropharnyx and anterior nares, due to difficulty in obtaining appropriate cultures from these sites. The numbers of yeasts and bacteria in these cultures were quantitated, and all of the yeasts isolated were identified. The numbers of yeasts present at each of the sites described above were expressed as an absolute number and as a percent of the total number of microorganisms isolated from each site. Blood cultures were obtained at the time of initiation of TPN therapy; all microorganisms isolated from these cultures were identified. Urine cultures for Candida were also obtained. All of the cultures described above were repeated during episodes of suspected septicemia and at the termination of TPN therapy.

At the time of catheter removal, the bacteria and yeasts present on a proximal 3 cm segment of each catheter beginning 1 cm inside of the former skin catheter interface (intradermal segment) and a 3 cm segment of the catheter tip (intravascular segment) were eluted with 0.1 M phosphate-buffered saline, pH 7.4, and quantitated. All bacteria and yeasts isolated from the intradermal and intravascular segments of the catheter were identified by standard methods and were expressed as absolute numbers.

The results of the quantitative cultures for Candida, obtained prior to insertion of the 10 catheters, are presented in Table 9. Two rectal, 6 mouth, and 2 burn wound cultures were positive for Candida; all catheter insertion site cultures were negative for Candida. The species of Candida isolated from all cultures, except the mouth culture of Patient 5, was albicans; the species of Candida isolated from this mouth culture was tropicalis. Candida were present in the mouth in both instances when Candida were isolated from the rectum and, in one of the two cases, when Candida were isolated from the burn wound. However, no correlation was demonstrated between the absolute number of Candida present in the mouth or the number of Candida relative to the total number of microorganisms and the number or percent of Candida present at the other sites.

Cultures obtained upon termination of TPN therapy are presented in Table 10. Six rectal, 9 mouth, 4 burn wound, and 5 catheter insertion site cultures were positive for Candida. All sites which were colonized with Candida at the initiation of TPN therapy were positive for yeasts when therapy was terminated, and some of the sites which were negative

Table 9. Numbers of *Candida* Colonizing the Rectum, Mouth, Burn Wound, and Catheter Insertion Site of the Patients at the Start of TPN Therapy.

Patient No.	Site Cultured			
	Rectum	Mouth	Burn Wound	Catheter Insertion Site
1				
Catheter I	2.4[a] (<0.1%)[b]	6.3 (4%)	NCI[c]	NCI
Catheter II	NCI	5.7 (0.4%)	2.8 (50%)	NCI
2	NCI	NCI	NCI	NCI
3	3.6 (<0.1%)	4.0 (9%)	NCI	NCI
4	NCI	2.8 (<0.1%)	NCI	NCI
5	NCI	6.0[d] (83%)	NCI	NCI
6	NCI	NCI	NCI	NCI
7				
Catheter I	NCI	4.2 (0.3%)	NCI	NCI
Catheter II	NCI	NCI	1.0 (<0.1%)	NCI
8	NCI	NCI	NCI	NCI

[a] Log_{10} c.f.u. per site.

[b] Percent of total number of microorganisms present at the site.

[c] NCI = no *Candida* isolated.

[d] *C. tropicalis* was isolated from this site. All other isolates were *C. albicans*.

Table 10. Numbers of *Candida* Colonizing the Rectum, Mouth, Burn Wound, and Catheter Insertion Site of the Patients at the Termination of TPN Therapy.

Patient No.	Site Cultured			
	Rectum	Mouth	Burn Wound	Catheter Insertion Site
1				
Catheter I	1.7[a] (<0.1%)[b]	5.5 (1%)	1.7 (<0.1%)	1.9 (0.8%)
Catheter II	2.2 (<0.1%)	6.1 (2%)	2.2 (60%)	2.5 (13%)
2	NCI[c]	3.0 (0.2%)	NCI	NCI
3	3.1 (<0.1%)	3.6 (4%)	NCI	NCI
4	NCI	3.4 (<0.1%)	NCI	3.3 (64%)
5	2.9[d] (<0.1%)	4.9[d] (15%)	NCI	NCI
6	NCI	NCI	NCI	NCI
7				
Catheter I	2.6 (<0.1%)	4.0 (<0.1%)	NCI	NCI
Catheter II	NCI	1.0 (<0.1%)	1.4 (<0.1%)	1.3 (<0.1%)
8	1.0 (<0.1%)	2.0 (<0.1%)	5.8[d] (74%)	4.1[d] (85%)

[a] Log_{10} c.f.u. per site.

[b] Percent of total number of microorganisms present at the site.

[c] NCI = no *Candida* isolated.

[d] *C. tropicalis* was isolated from these sites. All other isolates were *C. albicans*.

prior to TPN therapy were positive upon termination. Conversion from negative to positive cultures for Candida was most prevalent at the catheter insertion sites. In every case that the catheter insertion site was colonized with Candida, the yeasts were also present in the mouth. However, there were also patients with positive mouth cultures, who did not have positive insertion site cultures, and no correlation was demonstrated between absolute or relative numbers of Candida in the mouth and the presence of Candida at the insertion sites. The species of Candida isolated from all the patients, except Patients 5 and 8, was albicans. In Patient 5, the yeasts isolated were C. tropicalis, and in Patient 8, C. albicans was isolated from the rectum and mouth, while C. tropicalis was isolated from the burn wound and catheter insertion site. This observation may have been due to exogenous contamination of the burn wound or insertion site with C. tropicalis. Alternatively, C. tropicalis may have been present in numbers too few to quantitate in the mouth and rectal cultures.

Candida were isolated from catheters removed from 3 of the 5 insertion sites colonized with Candida (Table 11). C. albicans was isolated from 2 catheters and C. tropicalis was isolated from 1 catheter. Candida were isolated from the intradermal segment of two of the catheters and from both the intradermal and intravascular segments of the third catheter. Pseudomonas fluorescens was also isolated from both segments of one of these catheters and was also present at the insertion site. Of the remaining 7 catheters, 3 were positive for S. aureus alone or in combination with diphtheroids or Enterobacter cloacae. One was positive for P. aeruginosa, and 3 were sterile. Microorganisms present on the catheters were isolated concurrently from the insertion sites, with the exception of 1 catheter colonized with diphtheroids.

Two of the 3 patients with positive Candida catheter cultures had positive blood cultures for Candida during TPN therapy; bacteremia was also documented in these patients (Table 12). Both developed systemic candidosis as documented by multiple positive blood cultures which did not clear upon catheter removal, a four-fold rise in antibody titer against Candida antigen determined by counterimmunoelectrophoresis, and evidence of Candida endophthalmitis. The other patient, from which Candida were isolated from the catheter, did not develop bacteremia, candidemia, or other evidence of systemic candidosis. However, suppurative pyelonephritis was observed at necropsy. Patients with catheter cultures positive for S. aureus developed S. aureus bacteremia during TPN therapy. The patient with catheter cultures positive for P. aeruginosa developed bacteremia caused by P. aeruginosa and other microorganisms. The 3 patients with negative catheter cultures had negative blood cultures during TPN therapy.

Of the 2 patients who developed systemic candidosis, 1 had positive urine cultures for Candida at the initiation and termination of TPN therapy (Patient 8) and the other had positive urine cultures for Candida upon termination but not initiation of TPN therapy (Patient 4). Positive urine cultures for Candida were documented in only 1 other patient; this patient was the one who developed a positive catheter

Table 11. Surface Quantitative Culture Results of the Catheter Insertion Sites and Catheters at the Termination of TPN Therapy.

Patient No.	Catheter Insertion Site	Catheter	
		Intradermal Segment	Intravascular Segment
1			
Catheter I	Pseudomonas fluorescens (2.0)[a] Staphylococcus aureus (4.4) Candida albicans (1.9)	Staphylococcus aureus (3.9)	Staphylococcus aureus (2.7)
Catheter II	Pseudomonas fluorescens (3.3) Candida albicans (2.5)	Pseudomonas fluorescens (2.7) Candida albicans (2.4)	Pseudomonas fluorescens (1.8)
2	N.G.[b]	N.G.	N.G.
3	Staphylococcus aureus (1.8)	N.G.	N.G.
4	Candida albicans (3.3) Pseudomonas aeruginosa (2.9) Proteus mirabilis (2.5)	Candida albicans (3.3)	N.G.
5	N.G.	N.G.	N.G.
6	Pseudomonas aeruginosa (6.1) Staphylococcus epidermidis (2.3)	Pseudomonas aeruginosa (3.4)	Pseudomonas aeruginosa (2.9)
7			
Catheter I	Staphylococcus aureus (5.6)	Staphylococcus aureus (2.0) diphtheroids (4.1)	Staphylococcus aureus (2.3) diphtheroids (2.9)
Catheter II	Staphylococcus aureus (6.5) Enterobacter cloacae (6.7) Actinobacter calcoaceticus (4.7) Candida albicans (1.3)	Staphylococcus aureus (4.2) Enterobacter cloacae (3.0)	Staphylococcus aureus (3.5) Enterobacter cloacae (2.3)
8	Candida tropicalis (4.1) Pseudomonas aeruginosa (2.2) Staphylococcus aureus (1.7)	Candida tropicalis (2.4)	Candida tropicalis (1.2)

[a] c.f.u. expressed in Log $_{10}$.

[b] N.G. = no growth.

Table 12. Temporal Sequence of Positive Blood Cultures During TPN Therapy in the Patients.

Patient No.	Postburn Days of TPN Therapy	Positive Blood Cultures	
		Postburn Day	Microorganism Isolated
1	7 - 14	12, 14	Staphylococcus aureus
	27 - 40	39	N.G.[a]
2	3 - 7	5, 6	N.G.
3	13 - 15	14	N.G.
4	23 - 31	27	Staphylococcus aureus
		28-30, 35	Candida albicans
5	14 - 22	15, 17, 18	N.G.
6	9 - 14	9	Staphylococcus aureus, Actinobacter calcoaceticus
		11, 12	Staphylococcus aureus, Pseudomonas aeruginosa
		13	Staphylococcus aureus
7	17 - 20	16, 17, 18	Staphylococcus aureus
		21	N.G.
	22 - 26	24	Staphylococcus aureus
		26, 28, 29	N.G.
8	20 - 25	20	Pseudomonas aeruginosa
		20,26,28,41	Candida tropicalis

[a] N.G. = no growth.

culture for Candida who did not develop candidemia or bacteremia. Urine cultures were positive for Candida in this patient at both initiation and termination of TPN therapy.

In our next experiments, two ELISA methods were evaluated for their sensitivity and accuracy in detecting Candida antigens to aid in the early diagnosis of systemic candidosis in burned patients. The ELISA methods which were evaluated were the double-antibody sandwich technique (85) and the inhibition technique (86). The double-antibody sandwich technique involves the coating of cuvettes with the IgG fraction of specific antiserum to known antigen, then test antigen, and then alkaline phosphatase-labeled IgG fraction of homologous antiserum. The inhibition technique involves the coating of cuvettes with known antigen, then residual antibodies (after incubation of homologous antiserum with test antigen), and then alkaline phosphatase-labeled anti-IgG.

The Candida antigens selected for use in the ELISA methods were homogenate (H) antigen, cytoplasmic (C) antigen, and peptidoglucomannan (PGM) antigen. The 3 antigens were purified from C. albicans CR-1473 as follows: (a) The H antigen was prepared by sonication of washed cells followed by removal of solids by centrifugation (87); (b) the C antigen was prepared by sonication of washed cells and removal of mannan from the supernatant by chromatography on Con A-Sepharose (88); (c) the PGM antigen was prepared by alkaline hydrolysis of purified cell walls (89).

Antisera to the 3 antigen preparations were prepared in rabbits, and the specificity of the antisera was tested by Ouchterlony analysis. Antiserum from rabbits immunized with the PGM antigen formed 2 precipitin bands with all 3 antigen preparations. Antisera from rabbits immunized with the C or H antigen formed multiple precipitin bands with the C and H antigen preparations and 2 precipitin bands with the PGM antigen. Further Ouchterlony analysis of the anti-C serum with the PGM and C antigen preparations demonstrated that one of the multiple bands which formed between the anti-C serum and C antigen showed complete identity with one of the 2 bands which formed between the anti-C serum and PGM antigen. These results suggested that the C antigen preparation was contaminated with PGM, and therefore serum from rabbits immunized with the C antigen preparation contained contaminating antibodies directed against the PGM antigen.

In an attempt to remove the contaminating antibodies, the anti-C serum was adsorbed with purified mannan covalently linked to epoxy-activated Sepharose 6B (90); the mannan was purified from C. albicans CR-1473 by the method of Peat (91). In addition, the C antigen preparation was further purified by a second chromatography over Con A-Sepharose. By Ouchterlony analysis, the adsorbed anti-C serum contained no detectable antibody directed against the PGM antigen, and the further purified C antigen contained no detectable PGM antigen.

Our next experiments were performed to determine the optimal concentrations of the IgG fractions required for coating cuvettes for

detection of antigen in the double-antibody sandwich ELISA method. The IgG fractions of the antisera were prepared by ammonium sulfate precipitation. Polystyrene cuvettes were incubated at 37° C for 120 minutes with increasing concentrations of the immune IgG fractions (1 ng/ml to 100 µg/ml) in 0.05 M carbonate buffer, pH 9.6 (coating buffer). The cuvettes were then washed 7 times with 0.15 M NaCl containing 0.05% Tween 20, pH 7.2 (NaCl-Tween). The IgG coated cuvettes were next incubated at 37° C for 120 minutes with alkaline phosphatase-labeled goat anti-rabbit IgG (diluted 1:500 in 0.1 M phosphate-buffered saline containing 0.05% Tween 20, pH 7.2 (PBS-Tween). The cuvettes were washed 7 times with NaCl-Tween, and P-nitrophenyl phosphate (substrate) was added. The cuvettes were incubated at 37° C for 90 minutes, and the reaction was stopped by the addition of 0.5 N NaOH. The absorbance was read at 405 nm and compared to the absorbance of the negative controls, in which cuvettes were coated with bovine serum albumin (BSA). The minimum concentration of each immune IgG fraction which yielded an absorbance of 3 times the absorbance of the respective negative control was selected for cuvette coating. For all three IgG fractions, this concentration was found to be 50 µg/ml.

We next determined the optimal dilutions of the alkaline phosphatase-labeled immune IgG fractions required for detection of the antigens in the double-antibody sandwich ELISA method. The immune IgG fractions were covalently linked to alkaline phosphatase by the method of Engvall and Perlman (92). Cuvettes were coated with 50 µg/ml of non-labeled immune IgG fractions as described above. The cuvettes were then incubated at 37° C for 120 minutes with the homologous Candida antigen at a concentration of 1 µg/ml in PBS-Tween. The cuvettes were washed, and dilutions of the homologous alkaline phosphatase-labeled immune IgG fraction in PBS-Tween were added. The cuvettes were incubated at 37° C for 120 minutes. After the cuvettes were washed again, substrate was added and the cuvettes were incubated at 37° C for 90 minutes. The reaction was stopped by the addition of 0.5 N NaOH. The absorbance was determined at 405 nm and compared to the absorbance in the negative controls, in which cuvettes were coated with BSA and buffer was substituted for the antigen. The minimum dilution of each alkaline phosphatase-labeled immune IgG fraction which yielded an absorbance of 3 times the absorbance of the respective negative control was selected for detection of antigen in the double-antibody sandwich method. These dilutions were found to be 1:50, 1:100, and 1:100 for the IgG fractions of antisera to the PGM, C, and H antigens respectively.

Experiments were next performed to determine the sensitivity of the double-antibody sandwich ELISA method for detection of the 3 C. albicans antigens. Cuvettes coated with non-labeled immune IgG fractions were incubated at 37° C for 120 minutes with dilutions of the homologous Candida antigen preparation dissolved in PBS-Tween. After washing, the homologous alkaline phosphatase-labeled IgG fraction diluted in PBS-Tween was added to the cuvettes. The cuvettes were incubated at 37° C for 120 minutes and washed. Substrate was added and allowed to incubate at 37° C for 90 minutes, at which time the reaction was stopped by the addition of 0.5 N NaOH. The absorbance was read at 405 nm and compared

to the absorbance in the negative controls, in which cuvettes were coated with BSA and buffer was substituted for the antigens. The 3 antigen-antibody assay systems were only capable of detecting 100 ng of antigen. The apparent lack of sensitivity was related to the high background absorbance of the negative controls (0.59 ± 0.12).

In our next series of experiments, the sensitivity of the ELISA-inhibition technique for the detection of Candida antigens was investigated. The optimal dilutions of the antigens required for coating cuvettes was determined as follows: (a) Cuvettes were incubated at 37° C for 120 minutes with dilutions of the antigens dissolved in coating buffer and then washed 7 times with NaCl-Tween; (b) each antigen coated cuvette was incubated at 37° C for 120 minutes with the homologous rabbit antiserum (diluted 1:1000 in PBS-Tween) and then washed; (c) alkaline phosphatase-labeled goat anti-rabbit IgG (diluted 1:500 in PBS-Tween) was added; the cuvettes were incubated for 120 minutes at 37° C and then washed; (d) substrate was added and the cuvettes were incubated for 90 minutes at 37° C; the reaction was stopped by the addition of 0.5 N NaOH; (e) the absorbance was read at 405 nm and compared to the absorbance in the negative control, in which cuvettes were coated with BSA. The minimum concentration of antigen which yielded an absorbance of 3 times the absorbance of the negative control was selected to coat cuvettes for use in the ELISA-inhibition technique. These concentrations were found to be 1 μg/ml, 5 μg/ml and 10 μg/ml for the PGM, C, and H antigens respectively.

Dilutions of each of the Candida antigens in PBS-Tween were next incubated at 4° C for 16 hours with a known concentration of the homologous antiserum. An aliquot of the reaction mixture was transferred to cuvettes coated with the homologous antigen preparations. The cuvettes were incubated at 37° C for 120 minutes and then washed with NaCl-Tween. Alkaline phosphatase-labeled goat anti-rabbit IgG (diluted 1:500 in PBS-Tween) was added, and the cuvettes were incubated for 120 minutes at 37° C and then washed. Substrate was added, and the cuvettes were incubated at 37° C for 90 minutes. The reaction was stopped by the addition of 0.5 N NaOH. The absorbance was read at 405 nm and compared to the absorbance in the negative controls, in which cuvettes were coated with BSA and buffer was substituted for antigen in the primary incubation mixture. The percent inhibition was calculated by the formula $100 \times [1-(\text{absorbance in the test mixture/absorbance in the control})]$. The 3 antigen-antibody assay systems were capable of detecting 1 ng/ml of antigen dissolved in PBS-Tween. The sensitivity of the assay systems was shown to be identical, when the antigens were dissolved in pooled normal human serum. A representative ELISA-inhibition curve using PGM antigen dissolved in pooled normal human serum is presented in Figure 10.

Multiple sera from each of the 8 burned patients receiving TPN therapy were next tested by the ELISA-inhibition technique to determine the presence of the 3 Candida antigens. PGM antigen was detected in the sera of 4 of the 8 patients, and H antigen was detected in the sera of 3 of these patients (Table 13). None of the patients' sera contained detectable C antigen. Of the 4 patients in which PGM antigenemia was de-

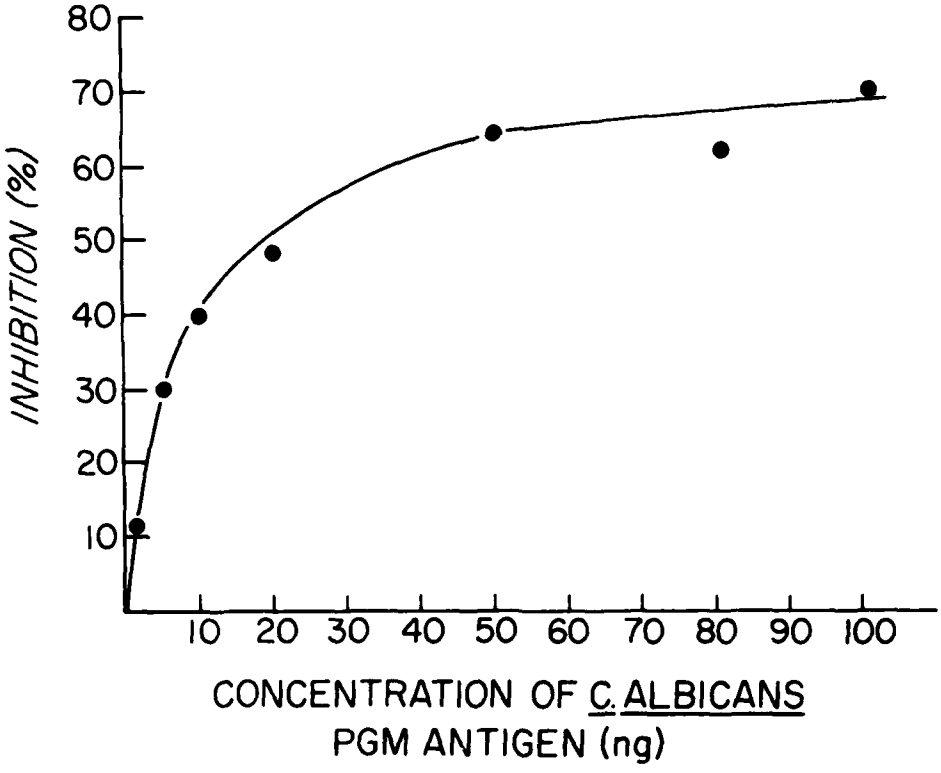

Figure 10. ELISA-inhibition of binding of anti-PGM antibodies to PGM coated cuvettes by prior incubation of the antibodies with increasing concentrations of PGM antigen dissolved in pooled normal human serum.

Table 13. Detection of *Candida* Antigens in the Patients' Sera by the ELISA-Inhibition Technique.

Patient No.	Days Postburn	Concentration of Antigen in Serum (ng)		
		Peptidoglucomannan	Homogenate	Cytoplasmic
1	7	0	0	0
	27	55	30	0
	39	52	25	0
2	3, 7	0	0	0
3	7, 13, 15	0	0	0
4	6	<5	0	0
	13, 23	0	0	0
	30	52	35	0
5	6, 14, 22	0	0	0
6	5, 9, 14	0	0	0
7	7, 17	0	0	0
	22	15	<5	0
	26	10	0	0
8	5	0	0	0
	20	0	0	0
	24	29	18	0
	46	55	35	0

tected, 2 developed systemic candidosis, and 1 had a positive catheter culture for Candida. The other patient had positive cultures for Candida in the mouth, rectum, burn wound, and catheter insertion site, but not on the catheter. In all of these patients, PGM antigenemia was detected after the initiation of TPN therapy and persisted until TPN therapy was terminated. In the one patient in which serum was collected after discontinuation of TPN therapy, PGM antigenemia was detected during that time; this was one of the patients with systemic candidosis. H antigenemia was detected concurrently with PGM antigenemia in the 2 patients who developed systemic candidosis and in the patient with the catheter positive for Candida. H antigenemia was not detected in the patient without catheter colonization, but who had multiple other sites colonized with Candida. These results suggest that H antigenemia correlated with Candida catheter colonization and systemic candidosis.

2. Discussion

The preliminary results presented in this report suggest that a primary portal of entry for Candida during the development of systemic candidosis in burned patients receiving TPN therapy is the subcutaneous tract created by intravascular catheters. Systemic candidosis, occurring during TPN therapy in 2 of 8 burned patients, was associated with positive catheter cultures for Candida and Candida antigenemia. In a third patient with similar numbers of Candida on the catheter, blood cultures were negative suggesting that bloodstream invasion did not occur. However, urine cultures were positive for Candida, and Candida antigenemia was demonstrated. In addition, suppurative pyelonephritis was reported at necropsy. Thus, disseminated disease might have been present antemortem, but was not diagnosed. The importance of direct access to the bloodstream for the development of invasive disease by Candida was also suggested by the observation that no patient with colonization limited to the gastrointestinal tract or burn wound became candidemic or developed evidence of systemic candidosis. The role of the catheter as a portal of entry for Candida is further supported by the clinical observation of an increased incidence of Candida infections associated with the use of intravascular catheters (12-15).

A prerequisite for invasion of the catheter appeared to be colonization of the catheter insertion site with a threshold number of Candida. The 3 patients with positive catheter cultures for Candida had from 3.1×10^2 to 1.3×10^4 Candida colonizing the insertion site. Catheter colonization was not demonstrated in 2 other patients with 2.0×10^1 to 8.0×10^1 Candida at the insertion site. Thus, it appears that greater than 3.0×10^2 Candida may be required before catheter colonization and the threat of bloodstream invasion occurs. Our results suggest that quantitative insertion site cultures may be an effective means of monitoring patients for possible catheter colonization with Candida, as well as other microorganisms, and for determining when catheter removal because of infectious complications would be most appropriate.

The mouth was a primary reservoir for Candida in the burned patients studied. Cultures of the mouth were positive for Candida in

60% of the cases at the initiation of TPN therapy and in 90% of the cases when TPN therapy was discontinued. The high incidence of Candida colonization of the mouth was probably related to antibiotic therapy, since all 8 patients had been treated with broad spectrum antibiotics within 7 days of the start of TPN therapy, and 7 of these patients were receiving antibiotics concurrently with TPN therapy. This concept is also supported by the reports of other investigators of similar rates of colonization of the mouth with Candida in patients receiving antibiotic therapy (11,18).

In all but one instance that the rectum, burn wound, or catheter insertion site was colonized with Candida, the yeasts were also present in the mouth. However, not all patients who had Candida present in their mouths developed colonization of the burn wound, catheter insertion site, or rectum. The presence of Candida at these sites did not correlate with either the absolute number of Candida in the mouth or the number of Candida relative to the total number of microorganisms. Thus, no definitive conclusion regarding the role of mouth colonization with Candida and colonization of other body sites can be made. However, it seems likely that the drooling of saliva by these critically ill patients onto the burn wound or catheter insertion site may have resulted in colonization of these areas. Alternatively, hospital personnel may have been the vehicle for inoculation of these areas with Candida during the performance of routine mouth care. Suppression of yeast growth in the mouth by the application of topical antifungal agents may reduce the potential for spread of Candida to other body sites and thereby decrease the potential for the development of systemic candidosis.

The ELISA-inhibition technique using a homogenate antigen of C. albicans was found to be an accurate tool for documenting Candida antigenemia in patients with systemic candidosis or catheter colonization with Candida. The use of a peptidoglucomannan antigen in this assay system detected Candida antigenemia not only in patients with catheter colonization, but also in one patient who had positive burn wound, mouth, and rectal cultures without catheter colonization or positive blood cultures. The increased specificity of the assay using homogenate antigen as compared to the peptidoglucomannan antigen for the documentation of Candida antigenemia associated with catheter colonization may have been related to the fact that the former antigen preparation contained intracellular or cell wall antigens which were not present in the latter. Alternatively, peptidoglucomannan may have been present in a lower concentration in the homogenate antigen, and this concentration was optimal for the documentation of antigenemia associated with catheter colonization with Candida. In support of this hypothesis is the observation that the use of a cytoplasmic antigen in the assay system, presumably containing all of the antigens in the homogenate antigen preparation except peptidoglucomannan, failed to detect antigenemia in the patients with systemic candidosis or in the patient with Candida catheter colonization. Further studies on larger numbers of patients will be required to determine both the specificity and predictive value of the ELISA-inhibition technique using the homog-

enate and peptidoglucomannan antigens in the early diagnosis of systemic candidosis in burned patients.

V. CONCLUSIONS

A. Serum-mediated inhibition of PMN bactericidal activity

1. Sera from 3 of 12 burned patients obtained during 13 to 56 days postburn inhibited the bactericidal activity of normal human PMNs when tested at a physiologic concentration.

2. Decreased PMN bactericidal activity was not caused by deficient opsonization of the bacteria by the burn sera.

3. The burn sera inhibited the phagocytic process and thereby reduced the number of bacteria available for intracellular killing.

4. The inhibitory effect of the burn sera on phagocytosis was not dependent on bacterial surface properties.

5. The inhibitory effect resulted from a direct interaction of the burn sera with the leukocytes, which was not associated with cell death and was not reversed by washing of the leukocytes.

6. The burn serum inhibitor of PMN phagocytosis was non-dialyzable.

7. The occurrence of the burn serum inhibitor of PMN phagocytosis was not related to total burn size nor to the amount of third degree burn injury.

B. Reduction in alternative complement pathway mediated C3 conversion

1. Reduction in alternative complement pathway mediated C3 conversion occurred in burned patients with total burn sizes of 20% or greater.

2. The most marked reduction in alternative pathway mediated C3 conversion occurred in patients with large full-thickness injuries, who had infectious complications during their clinical course.

3. The abnormality was not, however, more severe in patients with pneumonia and bacteremia in comparison to patients with bacteremia only and was not predictive of fatal outcome resulting from infectious complications.

4. Dialysis of the burn sera corrected the reduction in alternative pathway mediated C3 conversion presumably by removing a low molecular weight serum inhibitor.

5. The burn serum inhibitor of alternative pathway activity was not recovered in protein-containing fractions of burn serum dialysates.

C. *Candida* colonization and serodiagnosis

1. The subcutaneous tract created by the central catheter was the portal of entry of *Candida* in the 2 burned patients who developed systemic candidosis while receiving TPN therapy.

2. The catheter was the source of positive blood cultures in 6 of the 7 patients who had catheters colonized with Candida or bacteria.

3. Microorganisms colonizing the catheter insertion site were isolated from the 7 catheters which were positive for Candida or bacteria.

4. A threshold number of 3.0×10^2 Candida on the skin or burn wound at the site of catheter insertion was required for catheter colonization.

5. The mouth was the primary reservoir for Candida in the 8 burned patients treated with TPN.

6. Candida antigenemia was detected in the sera of 3 patients with catheters colonized with Candida by the ELISA-inhibition technique.

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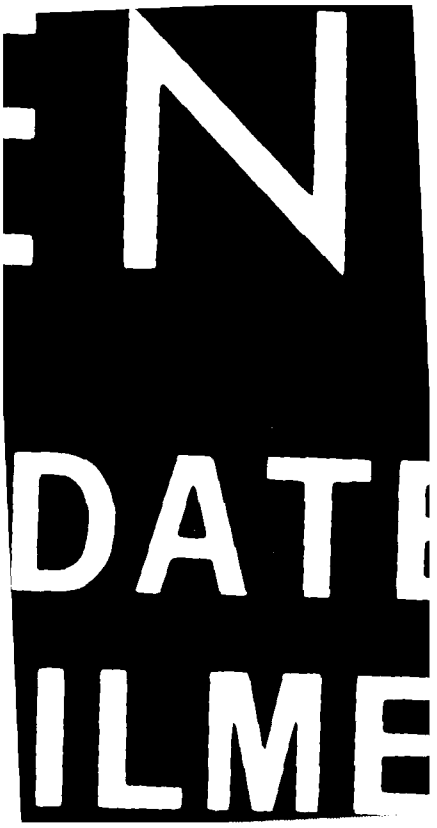
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